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Factors Promoting Human Papillomavirus Mediated Cervical Carcinogenesis

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Factors Promoting Human Papillomavirus Mediated Cervical Carcinogenesis

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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CONTENT

Approval Page iii

Acknowledgements iv

List of Figures viii

List of Tables ix

List of Abbreviations x

Abstract of the Dissertation xiii

1. Introduction 1

Mechanisms of Viral Carcinogenesis 5

Direct Transformation through Expression of Viral Genes 6
Indirect Transformational Activities 9

DNA Damage and Viruses 10

DNA Damage and Virus Infection 11

Factors that Cause DNA Damage and that Contribute to Carcinogenesis 13

Inflammation and DNA Damage Induced by Virus Itself 13
Inflammation and DNA Damage induced by Co-Infections 14
Oxidative Stressors, Viruses and Cancer 15

Integration of Viral DNA into the Human Genome 18

Hepatitis B Virus (HBV) 21
Human Papillomaviruses (HPVs) 24
Merkel Cell Polyomavirus (MCV) 29
5.4. Epstein-Barr Virus (EBV) 30

Prevention and Reduction of Risk Factors for Cancers 32

2. Chronic oxidative stress increases the integration frequency of foreign DNA and human papillomavirus 16 in human keratinocytes 34

Abstract 35

Keywords 36

Introduction....37
Materials and methods....40

Reagents....40
Cell culture....40
Transfections and transductions....41
Clonegenic assay....41
Immunoprecipitation....42
Flow cytometry assessment of ROS....42
Glutathione levels....43
Comet assessment of DNA damage....43
APOT (amplification of papillomavirus oncogene transcripts)....44
Quantitative Reverse Transcription PCR (qRT-PCR)....45

Results....46

E6*, but not E6, increased the level of OS and the integration frequency of plasmid DNA into U2OS cells....46
Increasing ROS results in increased integration frequency, while decreasing ROS results in decreased integration frequency....48
OS, induced by either overexpression of E6* or by glutathione depletion, increases DNA damage and the frequency of plasmid DNA integration into NOK cells....52
OS induced by glutathione depletion increases DNA damage in HCK cells containing episomal HPV16....57
Chronic OS increases the frequency of HPV16 genome integration in HCK cells containing episomal HPV16....60

Discussion....63

3. Identification of integration sites by Next Generation Sequencing (NGS) based analysis....69

Introduction....69
Materials and methods....70

Reagents....70
Cell culture....70
Preparing of HPV-specific SureSelect libraries....70
NGS data analysis....71

Results....71

NGS data showed exogenous factors increase integration rate of HPV 16 in HCK cells containing episomal HPV16....71

Discussion....74

4. Oxidative stress may influence susceptibility to cervical cancer: Cervical specimens display variability in levels of ROS and DNA damage 78

Abstract 79
Introduction 80
Material and methods 84

Preparation of cervical specimens 84
ROS analysis - cervical tissue 84
Isolation and culturing of normal primary cervical keratinocytes 85
ROS analysis – cultured keratinocytes 85
Immunoblot assays 85
DNA damage - 8-oxyguanine analysis 86
Statistics 86

Results 87

Collection and preparation of cervical TZ and EC specimens 87
ROS levels in cervical TZ tissues differ within a three-fold range between patients 90
Culture of primary keratinocytes 93
Variability in ROS levels in cultured primary keratinocytes isolated from the TZ is higher than in keratinocytes isolated from the EC 95
ROS levels in tissue homogenates correspond to those observed in cultured keratinocytes 98
Low levels of Gpx1/2, an antioxidant enzyme, correlate with higher levels of ROS in TZ keratinocytes 100
High levels of 8-oxoguanine, a marker of DNA damage, correlate with higher levels of ROS 102

Discussion 105

5. Discussion 111

References 114

FIGURES

Figures Page

1. Integration into the host genome 20

2. E6*, but not E6, increased the level of OS and the integration frequency of plasmid DNA into U2OS cells 47

3. Increasing ROS results in increased integration frequency, while decreasing ROS results in decreased integration frequency 51

4. OS, induced by either overexpression of E6* or by glutathione depletion, increases DNA damage and the frequency of plasmid DNA integration into NOK cells 56

5. OS induced by glutathione depletion increases DNA damage in HCK cells containing episomal HPV16 59

6. Chronic OS increases the frequency of HPV16 genome integration in HCK cells containing episomal HPV16 62

7. Chronic OS induced by long term BSO treatment increases the integration rate 76

8. Verification of the candidate insertion sites 77

9. Representative specimen 89

10. ROS levels in cervical TZ tissues 92

11. Morphology of cultured keratinocytes isolated from EC and TZ 94

12. Cultured primary keratinocytes isolated from the TZ display higher levels of ROS than do keratinocytes isolated from the EC 97

13. ROS levels in cervical tissues mirror the ROS levels observed in the corresponding cultured keratinocytes 99

14. Decreased levels of GPX1/2 expression were observed in TZ keratinocytes as compared to EC keratinocytes 101

15. ROS and DNA damage levels in cervical keratinocytes closely mirror each other 104

16. Potential cofactors promote cervical carcinogenesis 113

TABLES

Tables Page

1. Oncogenic viruses and associated cancers 4
2. Deep sequence mapping and HPV integration across cell lines 73

ABBREVIATIONS

HPV	Human Papillomavirus
OS	Oxidative stress
ROS	Reactive Oxygen Species
KSHV	Kaposi's sarcoma herpesvirus
MCV	Merkel cell polyomavirus
EBV	Epstein-Barr virus
HBV	Hepatitis B virus
SV40	Simian virus 40
HTLV-1	Human T-lymphotropic virus-1
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HBx	HBV X gene
EBV	Epstein-Barr virus
ATL	Adult T-cell leukemia
MCC	Merkel cell carcinoma
hTERT	Telomerase reverse transcriptase
SSB	Single-strand breaks
DSB	Double-strand breaks
ATM	Ataxia telangiectasia-mutated
STAT3	Signal transducer and activator of transcription 3 protein
STD	Sexually transmitted diseases

COX-2	Cyclooxygenase-2
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
MLL4	Mixed-lineage leukemia 4
PARP-1	Poly(ADP-ribose) polymerase-1
CIN	Cervical intraepithelial neoplasia
NO	Nitric oxide
CFSs	Common fragile sites
NGS	Next Generation Sequencing
LCLs	Lymphoblastoid cell lines
HR HPVs	High-risk HPVs
BSO	L-Buthionine-sulfoximine
APOT	Amplification of Papillomavirus Oncogene Transcripts
NOK	Normal Oral Keratinocytes
HCK	Human Cervical Keratinocytes
PBS	Phosphate-buffered saline
FBS	Fetal bovine serum
pE6*	Plasmids encoding E6*
pE6 large	Plasmids encoding E6 large
pFlag	Plasmids encoding empty vector
H_2O_2	Hydrogen Peroxide
OH^-	Hydroxyl radicals

ROO^-	Peroxyl radicals
$O2^-$	Superoxide
DCFDA	5-(and-6)- carboxy-2',7'-dichlorodihydrofluorescein diacetate
DHE	Dihydroethidium
qRT-PCR	Quantitative Reverse Transcription
PGK	Phosphoglycerokinase
gamma GCS	gamma glutamyl cysteine synthetase
DDR	DNA Damage Response
ND10	Nuclear domain 10
TZ	Transformation zone
EC	Ectocervical
GR	α-glutathione reductase
SCJ	Squamocolumnar junction

ABSTRACT OF THE DISSERTATION

Factors Promoting Human Papillomavirus Mediated Cervical Carcinogenesis

by

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Dr. Penelope J. Duerksen-Hughes, Chairperson

High-risk human papillomavirus (HPV) is the causative agent of cervical cancer. Integration of the HPV genome into the host genome is a key event in cervical carcinogenesis, with oxidative stress (OS) likely playing a major role in promoting DNA damage, and subsequently, integration. In our current study, we demonstrated a chain of events leading from the induction of OS, to DNA damage, and then to viral integration. Induction of OS by either virus-mediated factors, such as expression of E6*, a splice variant of the E6 oncogene, or by exogenous factors led to DNA damage in normal oral keratinocytes and in cervical keratinocytes containing episomal HPV16. We found that OS increased the integration rate for both foreign DNA and HPV, while antioxidants reduced the integration frequency. We also demonstrated a significant variability in ROS levels in patient-derived cervical specimens, which may reflect differences in susceptibility to cervical cancer between women.

CHAPTER ONE

INTRODUCTION

Viruses are the causative agents of approximately 10%–15% of all cancers worldwide. Viruses that have been linked to carcinogenesis include several DNA viruses: Kaposi's sarcoma herpesvirus (KSHV), Merkel cell polyomavirus (MCV), Epstein-Barr virus (EBV), Human papillomavirus (HPV), hepatitis B virus (HBV) and the simian virus 40 (SV40), as well as at least two RNA viruses: human T-lymphotropic virus-1 (HTLV-1) and the hepatitis C virus (HCV). These viruses and their associated cancers are shown in Table 1.

The mechanism of virus transmission varies depending on the type of virus, the type of infection, its targets and the associated life cycle. Sexual transmission has been attributed to HBV (Fairley & Read, 2012), HCV (Tohme & Holmberg, 2010) and HPV (Burchell, Winer, de Sanjose, & Franco, 2006). HBV and HCV infections can be also acquired from contaminated blood transfusions (Buddeberg, Schimmer, & Spahn, 2008; Wilkins, Malcolm, Raina, & Schade, 2010) or can be transmitted through needle sharing between intravenous drug users (Hughes, 2000; Wilkins et al., 2010). Perinatal transmission of HBV (Kazim, Wakil, Khan, Hasnain, & Sarin, 2002) and HCV (Lam, Gotsch, & Langan, 2010) has been observed from mother to child during pregnancy or labor, and EBV infections can be transmitted through saliva from one individual to another (Cozad, 1996). SV40 has the potential to be introduced into humans by the poliovirus vaccine (Shah, 2007).

Progression to cancer as a result of infection with an oncogenic virus is usually a rare event. For example, the overall prevalence of high-risk HPV infection is 23% (Datta

et al., 2008). Most of these infections clear up without any intervention within a few months after acquisition, and about 90% clear within two years. Only 0.3%–1.2% of these initial infections will eventually progress to invasive cervical cancer, according to 2013 World Health Organization (WHO) data (Shulzhenko, Lyng, Sanson, & Morgun, 2014). Another example relates to the hepatitis B virus (HBV). The highest rate of infection was found in Sub-Saharan Africa and East Asia, where the prevalence of chronic hepatitis in the adult population is between 5%–10% according to 2014 WHO data ("WHO 2014 data: Hepatitis B,"). More than 90% of those infected people will recover and be completely cleared of the virus within six months, while less than 5% of infected people will develop chronic hepatitis. Twenty percent of chronic hepatitis B cases will progress to hepatic cirrhosis, and of these, only 5% will progress to hepatocellular carcinoma (HCC). In the case of another agent causing HCC, HCV, approximately 2%–3% (130–170 million) of the worldwide population has been infected with HCV (Negro & Alberti, 2011). According to 2014 data from the WHO, chronic HCV infection will develop in 55%–85% of infected persons, with the risk of hepatic cirrhosis being approximately 15%–30% within 20 years ("WHO 2014 data: hepatitis C,"). The transformation rate of cirrhosis to HCC is approximately 1%–3% per year (Rosen, 2011; Wilkins et al., 2010). According to WHO serological testing data, EBV, another oncogenic virus, is present in approximately 95% of adults worldwide, including those residing in the United States. However, only in very rare cases do these infections progress to Burkitt's lymphoma or nasopharyngeal carcinoma. Similarly, the prevalence of HTLV-I infection in some endemic areas is 15% ("Recommendations for counseling persons infected with human T-lymphotrophic virus, types I and II. Centers for Disease

Control and Prevention and U.S. Public Health Service Working Group," 1993), while adult T-cell leukemia (ATL) only develops in an estimated 2%–4% of these infected persons in endemic regions, and only where early childhood infection is common (Kondo et al., 1985; Murphy et al., 1989). Overall, these data indicate that infection with oncogenic viruses does not mean the obligatory development of cancer, although infected individuals can be considered an at-risk population for cancer development. Epidemiological studies demonstrate that risk factors for cancer include virus load, persistence of infection and duration of infection (C. J. Chen et al., 2006; Dalstein et al., 2003).

Although these viruses belong to different groups, display different etiologies, carry out a variety of life cycles, target different organs and utilize variable mechanisms to induce cancer, some common features of cancer development can be noted. Overall, these common features are related to virus-mediated and exogenously-derived factors that create favorable conditions for cancer promotion and progression.

Table 1. Oncogenic viruses and associated cancers
HTLV-1, human T-lymphotropic virus-1; KSHV, Kaposi's sarcoma herpesvirus; SV40, simian virus 40.

Virus	Genome	Associated Cancers
HTLV-1	Positive-strand, single-stranded RNA retrovirus	Adult T-cell leukemia (ATL) (Poiesz et al., 1980). ATL is a malignancy of CD4+ T-lymphocytes, which exhibits severe immunodeficiency and resistance to intensive chemotherapies (Yasunaga & Matsuoka, 2007).
HCV	Positive-strand, single-stranded RNA flavivirus	Some hepatocellular carcinoma (HCC) and possibly some lymphomas (Choo et al., 1989; Marcucci & Mele, 2011). The risk of HCC is 11.5- to 17-fold in HCV-infected patients (Donato, Boffetta, & Puoti, 1998; Donato et al., 2002).
KSHV	Double-stranded DNA herpesvirus	Kaposi's sarcoma, primary effusion lymphoma (Chang et al., 1994). Kaposi's sarcoma is the most frequent cause of malignancy among AIDS patients.
MCV	Double-stranded DNA polyomavirus	Merkel cell carcinoma (MCC) (Feng, Shuda, Chang, & Moore, 2008). MCC is a rare, but aggressive human skin cancer, and it typically affects the elderly, as well as immunosuppressed individuals.
EBV	Double-stranded DNA herpesvirus	Most Burkitt's lymphoma and nasopharyngeal carcinoma (Epstein, Achong, & Barr, 1964).
HBV	Partially double-stranded DNA hepadnavirus with retroviral features	Chronic infection with HBV has been linked to the development of HCC for over 30 years (Blumberg, Alter, & Visnich, 1965; Di Bisceglie, 2009).
HPVs	A group of circular, double-stranded DNA viruses (Flores & Lambert, 1997).	High-risk human papillomaviruses (HPV) 16 and HPV 18 (some other α-HPV types are also carcinogenic) are associated with cervical cancer, penile cancers and some other anogenital and head and neck cancers (Boshart et al., 1984; Durst, Gissmann, Ikenberg, & zur Hausen, 1983).
SV40	Polyomavirus of the rhesus macaque (Shah, 2007)	SV40 sequences are detected in 60% of human mesothelioma, a rare tumor related to exposure to asbestos (Carbone et al., 1994). It is also detected in brain tumors (Bergsagel, Finegold, Butel, Kupsky, & Garcea, 1992; H. Huang et al., 1999; Martini et al., 1996), osteosarcoma (Martini et al., 2002) and non-Hodgkin lymphoma (NHL) (Shivapurkar et al., 2002; Vilchez et al., 2002).

Mechanisms of Viral Carcinogenesis

Cellular transformation is a multi-step process that results in the transformation of healthy cells into cancer cells. It requires a progression of changes at the cellular, genetic and epigenetic levels that ultimately lead to the cellular changes necessary for uncontrolled cell division and formation of a malignant mass. Hanahan and Weinberg, in their landmark review "Hallmarks of Cancer", listed six essential alterations that must occur in a cell's physiology to cause malignancy, including self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg, 2000, 2011). More recently, two emerging hallmarks have been added to the list: deregulating cellular energetics and avoiding immune destruction (Hanahan & Weinberg, 2011). Cellular transformation induced by oncogenic viruses also adheres to Weinberg's hallmarks; in particular, limitless replicative potential, evasion of apoptosis and genome instability (Hanahan & Weinberg, 2000). Viruses, when functioning as carcinogenic agents, utilize a variety of carcinogenic mechanisms to transform human cells. One such mechanism is direct transformation, where the virus expresses viral oncogenes that can directly transform infected cells. Several viruses, including HPV, EBV, KSHV, SV40, HCV and HTLV, encode oncoproteins that employ several mechanisms to inactivate two of the major regulators of genome stability, cell viability and cell cycle; namely, the p53 and retinoblastoma proteins (pRB) (Levine, 2009).

Direct Transformation through Expression of Viral Genes

The tumor suppressor p53 is the product of the TP53 gene; it induces cell cycle arrest or apoptosis in response to cellular damage or insult, guards against genomic instability and plays a critical role in DNA repair (Collot-Teixeira, Bass, Denis, & Ranger-Rogez, 2004). Inactivation of p53 or depletion of its function in infected cells results in an accumulation of genomic mutations and DNA breaks, accumulating genomic instability, a loss of growth suppression and apoptosis, leading to promotion of cellular transformation (Collot-Teixeira et al., 2004; Weitzman, Lilley, & Chaurushiya, 2010). Perhaps the best-studied example of viral inactivation of p53 is provided by the E6 protein from high-risk (HR) HPV. The HR HPV E6 protein induces ubiquitin-mediated degradation of p53, resulting in disabling of the normal cellular response to many insults, including the DNA damage response (Scheffner, Werness, Huibregtse, Levine, & Howley, 1990). Another example of a virus with the ability to inactivate p53 is KSHV. The large multifunctional protein latency-associated nuclear antigen 1 (LANA1) expressed by KSHV interacts efficiently with p53, represses its transcriptional activity and inhibits p53-induced cell death (Friborg, Kong, Hottiger, & Nabel, 1999). Furthermore, the SV40 large T antigen (Tag) is well-known for its ability to bind and inactivate p53 (Carbone et al., 1997). In addition, it also plays a crucial role in cell-cycle derangement of human mesothelial cells (Levresse et al., 1997), followed by transformation of the cells (Butel & Lednicky, 1999). In the case of HCV, the NS5A viral oncoprotein is involved in apoptosis inhibition, signal transduction, transcription, transformation and the production of reactive oxygen species (ROS). In particular, NS5A has been shown to bind directly to p53 and to repress transcription of the tumor suppressor p21WAF1 in a p53-

dependent manner (Lan et al., 2002; Majumder, Ghosh, Steele, Ray, & Ray, 2001; Tsai & Chung, 2010).

In addition to p53, the tumor suppressor pRB is also a frequent target for oncogenic viruses. pRB regulates apoptosis during development, and its loss results in deregulation of growth and apoptosis (Hickman, Moroni, & Helin, 2002). In normal cells, pRB is hypophosphorylated in early G1, and becomes increasingly phosphorylated by cyclin D/CDK4/6 complexes as the cell moves towards S phase in response to a signal to divide. This results in the release of the E2F protein, which then activates the transcription of genes required for the S-phase transition (Hamid, Brown, & Gaston, 2009). E7, another HPV oncogene, mimics this process by binding to pRB and releasing the E2F protein (Dyson, Howley, Munger, & Harlow, 1989), thereby driving quiescent, infected cells back into a proliferative state in order to enable viral genome replication (Munger et al., 1989). In addition, the HR HPV E7 protein binds directly to E2F-1, leading to the activation of E2F-1-dependent transcription, and the affinity of E7 for E2F-1 appears to correlate with the oncogenic potential of the HPV (Hwang, Lee, Kim, Seo, & Choe, 2002). Therefore, HR HPV E6 works together with E7 to induce cellular genomic instability and mitotic defects, known contributors to carcinogenesis (Duensing et al., 2000). Another example of pRb inactivation is provided by the large T antigen (Tag) expressed by SV40. One domain of Tag, the LxCxE motif, binds to pRB and inactivates the protein (Ali & DeCaprio, 2001; De Luca et al., 1997). Despite the fact that adenoviruses do not cause cancer in humans, they can disrupt the pRB-E2F interactions via the activity of E1A, thus driving expression of viral transcription and inducing cell cycle progression (Seifried et al., 2008).

In addition to these two major targets, pRB and p53, several other molecules serve as common targets for oncogenic viruses. Telomerase, which is usually found in embryonic cells and is absent in somatic cells, is one such target. In normal cells, the telomeric regions shorten with each round of division (Campisi, Kim, Lim, & Rubio, 2001); inappropriate expression of telomerase can lead to immortalization. HR HPV E6 can activate the expression of the catalytic subunit of telomerase, telomerase reverse transcriptase (hTERT) (Klingelhutz, Foster, & McDougall, 1996; Veldman, Horikawa, Barrett, & Schlegel, 2001), in order to maintain telomeres through telomerase activation (Oh, Kyo, & Laimins, 2001; Veldman, Liu, Yuan, & Schlegel, 2003), and thereby contributing to immortalization. Another virus, EBV, encodes a principal oncoprotein, latent membrane protein 1 (LMP1), which is also able to activate hTERT (Terrin et al., 2008). HTLV-I expresses Tax protein, which is believed to be critical in the transformation of infected cells to adult T-cell leukemia (ATL). Tax is a 40-kDa trans-regulatory protein encoded by the tax gene located in ORF IV of the pX region (Jeang, Giam, Majone, & Aboud, 2004). Tax can repress the expression of hTERT by competition with c-Myc through a canonical c-Myc binding site within the hTERT promoter (Gabet et al., 2003). In addition to this function, it can also reprogram G1 to S progression through direct protein-protein binding, transcriptional induction/repression and post-translational modification (Jeang et al., 2004). Another target of oncogenic viruses is the tumor suppressor RASSF1A. The inactivation of this gene is correlated with the hypermethylation of its CpG-island promoter region, and the silencing of RASSF1A induces telomerase activity (Foddis et al., 2002). Tag of SV40 not only inactivates p53, but also inactivates the RASSF1A gene (Toyooka et al., 2002; Toyooka et al., 2001).

Several other signaling pathways involved in carcinogenesis are directly regulated by multifunctional viral oncoproteins. For example, the SV40 Tag can activate growth factor receptors, such as Met (Cacciotti et al., 2001), Notch-1 (Bocchetta, Miele, Pass, & Carbone, 2003) and IGF- 1R (Pass et al., 1996), leading to the activation of the ERK-kinase and AP-1 pathways that promote cell division and contribute to SV40-induced carcinogenesis (Carbone, Pass, Miele, & Bocchetta, 2003). In addition, NS5A, the HCV protein that depletes p53, can also interact with Bax and prevent apoptosis in a p53-independent manner (Chung, Sheu, & Yen, 2003). KSHV can express an interferon regulatory factor (IRF)-like signal-transduction protein, ORF K9, and this protein inhibits interferon-induced signaling pathways. This inhibition allows KSHV to overcome interferon-mediated antiviral activity and, thus, can contribute to host cell transformation (Moore & Chang, 1998). The LMP1 oncoprotein expressed by EBV (Mosialos et al., 1995), as well as Tax expressed by HTLV-I (Yasunaga & Matsuoka, 2007), both target the Nuclear Factor-κB (NF-κB) pathway. The NF-κB pathway plays a critical role in regulating the immune response to infection. NF-κB is a crucial mediator of inflammation-induced tumor growth and progression, as well as an important modulator of tumor surveillance and rejection (Karin & Greten, 2005).

Indirect Transformational Activities

In addition to these direct mechanisms underlying virus-induced carcinogenesis, virus-induced chronic infection and inflammation can also function as indirect transforming agents (zur Hausen, 2001). In fact, Colotta *et al.* in a 2009 paper in *Carcinogenesis* refer to inflammation as the seventh hallmark of cancer (Colotta, Allavena, Sica, Garlanda, & Mantovani, 2009). Evidence of the carcinogenic potential of

inflammation is provided by chronic inflammatory bowel diseases, such as chronic ulcerative colitis and Crohn's disease, which can lead to colon cancer, even in the absence of other factors (Coussens & Werb, 2002). Another well-known example is the chronic infection and inflammation induced by *Helicobacter pylori*, which results in stomach cancer worldwide (Ernst & Gold, 2000). Inflammation can also be caused by infection with viruses, thereby providing another linkage between infection and carcinogenesis. For example, chronic HBV or HCV infection can lead to hepatocellular carcinoma (HCC) through a process that induces cell death, regeneration, cirrhosis and, finally, cancer (Mason, Liu, Aldrich, Litwin, & Yeh, 2010; Seeger & Mason, 2000; Tsai & Chung, 2010). In many cases, the inflammation induced by chronic infection creates a microenvironment that favors expression of viral oncogenes. For example, in HBV-induced HCC, HBV is clonally integrated into host DNA, and the integrated HBV sequences encode HBV X (HBx) and/or truncated envelope pre-S2/S proteins in a large portion of the HCC. These oncoproteins are thought to participate in directly promoting transformation of hepatocytes to HCC (Tsai & Chung, 2010). Another example of the way in which virus infections can indirectly contribute to carcinogenesis is provided by cutaneous papillomavirus infections, which contribute indirectly to skin carcinogenesis by blocking apoptosis in cells exposed to ultraviolet light and, thus, permitting the survival of UV-damaged cells (Jackson & Storey, 2000).

DNA Damage and Viruses

p53 has been termed "the guardian of genome integrity." Its depletion or inactivation by virus proteins, as well as the inflammation produced as a result of chronic infection, both lead to an accumulation of point mutations, genomic instability and DNA

damage. DNA damage starts with a chemical modification to a base of DNA that induces a break in either one or both strands of the DNA (Hoeijmakers, 2009). In uninfected cells, DNA repair systems can recognize DNA damaged bases as abnormal structures and repair the damage prior to replication. The cell can call on several systems to repair DNA damage caused by both endogenous and exogenous factors, and these DNA repair pathways recognize both single-strand breaks (SSB) and double-strand breaks (DSB). SSB DNA repair mechanisms include direct repair, nucleotide excision repair, mismatch repair and base excision repair. The DSB repair mechanisms include homologous recombination and nonhomologous end joining (Basu, Yap, Molife, & de Bono, 2012). If the DNA damage cannot be completely repaired, this damage can lead to a deregulated cell cycle, genomic instability (Weitzman et al., 2010) and mutations associated with the development of cancer (Marnett, 2000; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006b). However, activation of DNA damage response and cell cycle regulation by virus proteins benefit virus production by providing an S-phase-like replication environment, preventing apoptosis and promoting episome maintenance (Weitzman et al., 2010).

DNA Damage and Virus Infection

During viral infections, the host must maintain genome integrity through the activation of its surveillance network for detecting and repairing DNA damage. Many viruses can employ direct and/or indirect mechanisms to activate DNA damage signaling pathways (Weitzman et al., 2010), and this DNA damage signaling can be activated either by virus infection itself or the expression of viral proteins. One example of DNA damage signaling activated by virus infection is seen in the case of EBV infections, where infection induces the cellular DNA damage response and activates the ataxia

telangiectasia-mutated (ATM) signal transduction pathway (Kudoh et al., 2005). ATM is the primary signal kinase activated after sensor proteins detect DNA damage. Autophosphorylation of ATM can activate downstream substrates, such as checkpoint kinase Chk2 and the DNA damage response protein, 53BP1. Inhibition of ATM and Chk2 significantly increases the transformation efficiency of EBV-infected primary B-cells (Nikitin et al., 2010). Another example is seen in the case of SV40 infection, where infection results in activation of ATM and endogenous ATM substrates (Shi, Dodson, Shaikh, Rundell, & Tibbetts, 2005). SV40 viral proteins can also induce the DNA damage response, as expression of the SV40 Tag protein activates the DNA damage response via binding to the mitotic spindle checkpoint kinase, Bub 1 (Hein et al., 2009). Activation of the DNA damage response is in the best interest of the virus, as it promotes SV40 viral DNA replication (Shi et al., 2005). In the case of HPV, expression of viral proteins from the HPV episome leads to ATM kinase activation, which, in turn, activates Chk2. Caspase activation due to Chk2 activity is necessary for productive viral genome amplification (Moody, Fradet-Turcotte, Archambault, & Laimins, 2007; Moody & Laimins, 2009). In any case, if the DNA damage repair system activated by the virus is not sufficiently efficient or if additional DNA damage induced by exogenous sources accumulates in the host genome, one side effect of this increased DNA damage in infected cells can be the integration of viral genomes to the host genome. In the case of HPV and HBV, viral genome integration is a major trigger point for the development of cancer. The role and importance of viral genome integration in cancer development will be discussed below.

Factors that Cause DNA Damage and Contribute to Carcinogenesis

Factors that induce DNA damage in infected cells can be divided into endogenous, *i.e.*, virus mediated, and exogenous factors.

Inflammation and DNA Damage Induced by Virus Itself

Inflammation is a primary immune response to infection by pathogens (Williams, Filippova, Soto, & Duerksen-Hughes, 2011). That process involves activation and directed migration of leukocytes from the venous system to the sites of infection; tissue mast cells also play a significant role (Coussens & Werb, 2002). A family of chemokines attract leukocytes, whose persistence at an inflammatory site is important in the development of chronic disease (Coussens & Werb, 2002).

Inflammation is referred to as a cancer "promoter", because it induces cell proliferation, recruits inflammatory cells, increases cellular levels of ROS, thereby leading to oxidative DNA damage, and reduces DNA repair (Coussens & Werb, 2002). The deregulation of cell death and/or repair programs results in DNA replication and proliferation in chronically-inflamed tissue (Coussens & Werb, 2002). Inflammation also causes resistance to apoptosis, secretion of pro-angiogenic and immunosuppressive factors, invasion and metastasis (Peebles et al., 2007) that are attributes of carcinogenesis. For example, the most common type of liver cancer results from chronic liver inflammation induced by either HBV or HCV (Brechot et al., 2010; Di Bisceglie, 1997). The enhanced DNA replication and DNA damage created during chronic inflammation increase the number of free DNA ends in host genomic DNA and promote HBV integration (Bonilla Guerrero & Roberts, 2005). In case of HCV, its core protein interacts with the signal transducer and activator of transcription 3 (STAT3) protein (T. Yoshida et

al., 2002), a transcription factor involved in mediating cytokine signaling (Bromberg & Darnell, 2000). This interaction results in enhanced proliferation and up-regulation of Bcl-XL and cyclin-D (T. Yoshida et al., 2002). In this way, chronic liver inflammation induced by HCV may alter the local cytokine profile and the balance between apoptosis and proliferation.

Inflammation and DNA Damage Induced by Co-Infections

Co-infections with certain sexually transmitted diseases (STD) cause cervical inflammation and increase the risk of cervical cancer in HPV-infected women (Castle & Giuliano, 2003; Castle et al., 2001; Williams et al., 2011). Furthermore, high levels of inflammatory mediators, such as cyclooxygenase (COX)-2, an enzyme responsible for prostaglandin formation, are observed in cervical cancer (G. E. Kim et al., 2004; Kulkarni et al., 2001). However, this inflammation is not thought to be primarily due to the HPV infection itself, but rather due to other factors, such as co-infections. One reason for the lack of HPV-induced inflammation is that HPV infects basal keratinocytes that are distant from immune centers and have short lifespans. In addition, the virus does not destroy the cells it infects, thereby avoiding the triggering of inflammation (Stanley, 2006). Co-infections that do trigger inflammation can be of either viral or bacterial origin. For example, studies have determined that co-infection with either Chlamydia trachomatis or HSV increase the risk of developing cervical cancer (Finan, Musharrafieh, & Almawi, 2006), as does infection with other STDs, such as Neisseria gonorrhoeae (Hawes & Kiviat, 2002). The inflammation induced by these co-infections can induce the generation of ROS, which can contribute to carcinogenesis by damaging DNA, as described below.

Oxidative Stressors, Viruses and Cancer

ROS and reactive nitrogen species (RNS) are charged free radicals that are primarily generated in mitochondria as by-products of aerobic respiration, cytochrome P450 activity and peroxisome function (Jezek & Hlavata, 2005). Under normal conditions, the pro- and anti-oxidant systems maintain ROS homeostasis. A lack of proper balance between these two sets of systems results in changes to cellular levels of ROS and can lead to oxidative stress (OS). In general, the sources of OS can be divided into two broad categories: exogenous and endogenous. Endogenous OS, as discussed above, is primarily derived from natural processes, such as cellular signaling, metabolic processes and inflammation (Altieri, Grillo, Maceroni, & Chichiarelli, 2008; Cadet, Douki, & Ravanat, 2010; De Bont & van Larebeke, 2004; Sedelnikova et al., 2010). Exogenous and environmental sources include ionizing radiation, such as X-, γ- and cosmic rays, α-particles from radon decay, oxidizing chemicals and UVA solar radiation. For example, ionizing radiation generates radicals, including superoxide, hydrogen peroxide and hydroxyl radicals (Riley, 1994), most of which are generated during the radiolysis of water. Of these, the hydroxyl radical is the most damaging species and produces mostly single-strand breaks (Wallace, 1998). Overall, radiation induces genetic instability and chromosomal rearrangements, and many of these rearrangements are similar to those found in human cancers (L. Huang, Snyder, & Morgan, 2003).

Chronic exposure to viral infections also induce the constant generation of free radicals (Georgakilas, Mosley, Georgakila, Ziech, & Panayiotidis, 2010), which can damage cellular biomolecules, including DNA. DNA damage produced by OS results in apurinic/apyrimidinic (abasic) DNA sites, oxidized purines and pyrimidines, and single- and

double-stranded DNA breaks (Kryston, Georgiev, Pissis, & Georgakilas, 2011). Therefore, the ROS- and RNS-induced oxidative and nitrative DNA damage that frequently occurs during inflammation can contribute to carcinogenesis (Kawanishi, Hiraku, Pinlaor, & Ma, 2006; Ohshima, Tatemichi, & Sawa, 2003).

A connection between OS, DNA damage and the incidence of hepatocellular carcinoma has been demonstrated by Hagen and colleagues (Hagen et al., 1994). The elevated level of ROS observed in chronic HBV-infected livers causes liver cell injury (Hagen et al., 1994; Shimoda et al., 1994) and may lead to an accumulation of repeated genetic damage and an increased risk for genomic alterations in infected hepatocytes. Moreover, cell necrosis and proliferation in chronic HBV infection in response to cell injury could allow for greater exposure of DNA to ROS and incomplete repair of DNA damage. All of these factors are predicted to increase the probability of the fixation of genetic and chromosomal abnormalities, thus causing mutations and enhancing the development of HCC (Hagen et al., 1994). Among the proteins encoded by HBV, the X gene product (HBx) is a protein that increases virus gene expression and replication to maintain viral infection by transactivating cellular promoters and enhancers (Feitelson et al., 2009; Keasler, Hodgson, Madden, & Slagle, 2007). HBx induces OS and contributes to liver disease pathogenesis associated with HBV infection (Waris, Huh, & Siddiqui, 2001).

High levels of ROS can directly regulate NF-κB activation. This was demonstrated in EBV-positive Burkitt's lymphoma cells, which have elevated ROS levels and altered NF-κB activation as opposed to EBV-negative Burkitt's lymphoma cells (Cerimele et al., 2005). EBV nuclear antigen-1 is the only viral protein expressed in all EBV-carrying malignancies, and it induces chromosomal aberrations and DNA DSBs

through increasing ROS production. This effect can be reversed by antioxidants (Gruhne, Sompallae, Marescotti, et al., 2009; Gruhne, Sompallae, & Masucci, 2009).

As opposed to several of these other viruses, infection with HPV does not in itself cause significant inflammation likely to lead to OS. However, expression of the smaller splice variant of E6, E6*, is able to increase ROS levels in oral keratinocytes. This ROS level increase is likely connected to the E6*-mediated decrease in the expression of the antioxidant enzymes, superoxide dismutase (SOD) 2 and glutathione peroxidase (GPx) 1/2 (Williams, Filippova, Filippov, Payne, & Duerksen-Hughes, 2014a). The increase in the level of oxidative stress due to E6* expression also resulted in an increase in DNA damage (Williams et al., 2014a).

Other environmental and lifestyle-related factors also contribute to the induction of oxidative stress in infected cells. Among the factors connected to lifestyles that can promote virus-induced tumorigenesis are alcohol consumption and tobacco smoking. For example, heavy alcohol intake, defined as ingestion of more than 50–70 g/day for prolonged periods, is a well-established HCC risk factor (El-Serag & Rudolph, 2007). Alcohol-induced oxidative stress plays an important role in the development of alcohol liver disease, and alcohol metabolism *via* the enzyme alcohol dehydrogenase results in increased ROS production, hepatocyte injury and apoptosis. Interestingly, all of these reactions could be blocked by the administration of antioxidants (Adachi & Ishii, 2002; Bailey & Cunningham, 2002).

Smoking is strongly associated with an increased risk of developing cervical cancer in HPV-positive women (Deacon et al., 2000; Plummer, Herrero, Franceschi, Meijer, Snijders, Bosch, de Sanjose, & Munoz, 2003). Smoking is known to induce

inflammation and oxidative stress (Tollefson et al., 2010), which may lead to DNA damage, integration and carcinogenesis, as discussed later.

Many studies have demonstrated that long-term use of oral contraceptives increases the risk of cervical cancer (Appleby et al., 2007; Moreno et al., 2002; Smith et al., 2003). The strongest evidence for this connection comes from the large pooled analysis of the International Agency for Research on Cancer (IARC) studies for the role of oral contraceptive use in HPV-induced carcinogenesis. Although "ever use" of oral contraceptives was only moderately associated with cancer risk, a strong dose-response relationship with increasing years of use was observed (Moreno et al., 2002).

High parity may also increase the risk of cervical cancer. One possible mechanism is that pregnancy maintains the transformation zone on the exocervix for many years (Autier, Coibion, Huet, & Grivegnee, 1996), thereby facilitating direct exposure to HPV and other cofactors. Hormonal changes induced by pregnancy, such as increased levels of estrogen and progesterone, may also modulate the immune response to HPV along with viral persistence and/or progression (Munoz et al., 2002; Sethi et al., 1998). High parity may also cause cervical trauma and cellular oxidative stress, thus leading to DNA damage and carcinogenesis (Castellsague & Munoz, 2003). In the IARC-pooled analysis, the odds ratio for cervical cancer in women with seven or more full-term pregnancies was four-fold higher than that in nulliparous women, and the risk increased linearly with an increasing number of full-term pregnancies (Munoz et al., 2002).

Integration of Viral DNA into the Human Genome

Cancer incidence is associated with integration of the viral genome for several oncogenic viruses, including HBV, HPV and MCV. It is not yet clear that integration of

EBV is a mechanism for carcinogenesis. The risk of virus integration depends on the level of DNA damage, because integration requires DSBs in both the host and virus DNA (M. Pett & Coleman, 2007) (see Figure 1). Furthermore, a major cause of DNA damage is OS, which can be triggered by viruses and enhanced by exogenous factors.

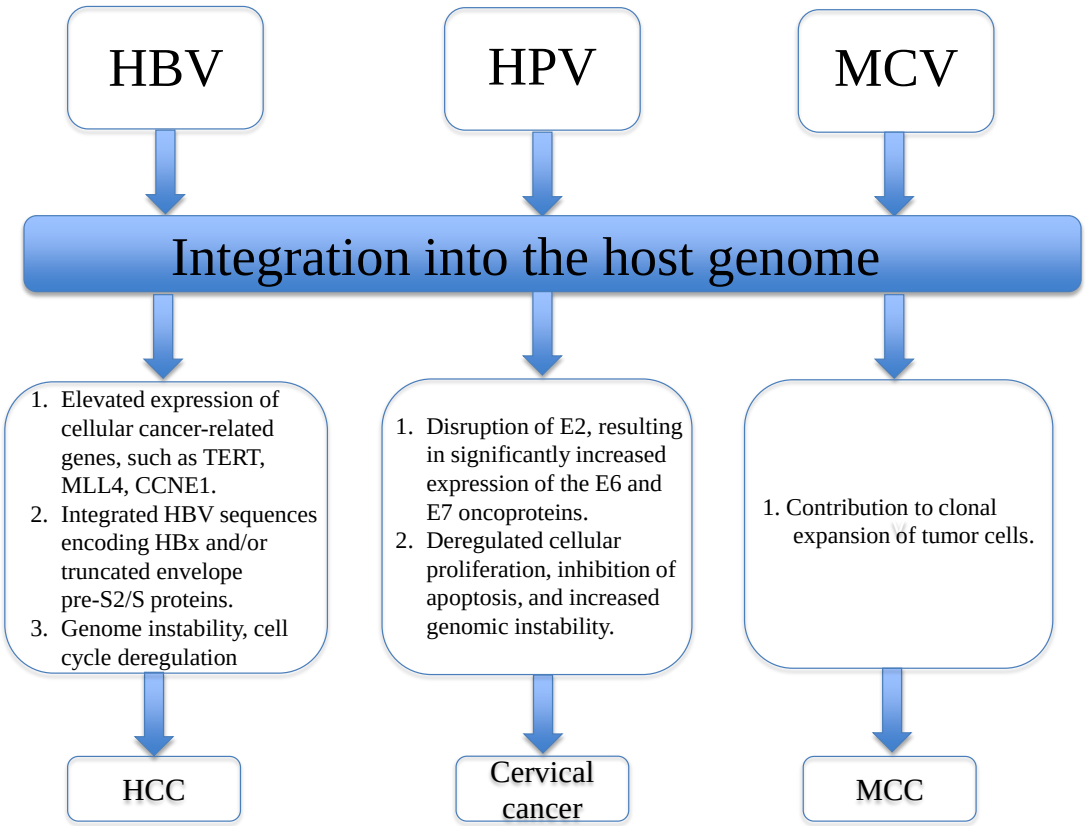

Figure 1. Integration into the host genome. Integration of HBV, HPV and MCV viral DNAs into the human genome induces cellular and viral responses and further contributes to carcinogenesis (HBV, hepatitis B virus; HPV, high-risk human papillomaviruses; MCV, Merkel cell polyomavirus; MCC, Merkel cell carcinoma; TERT, telomerase reverse transcriptase; MLL4, Mixed-lineage leukemia 4).

Hepatitis B Virus (HBV)

HCC is the fifth most common cancer and the third leading cause of cancer death worldwide (El-Serag & Rudolph, 2007), and up to 80% of HCC is attributable to either HBV or HCV infection (Perz, Armstrong, Farrington, Hutin, & Bell, 2006). HBV is a partially double-stranded DNA hepadnavirus with retroviral features. The risk of HCC is increased five- to 15-fold in chronic HBV carriers compared with the general population (El-Serag & Rudolph, 2007), and studies have shown that integration of the HBV genome into the cellular genome is present in over 85%–90% of HBV-related HCCs. However, the integrated form of HBV is also present in non-tumor tissue of patients with chronic HBV infections. Integration of the HBV genome into hepatocytes occurs during persistent HBV infection and precedes development of HCC (Brechot, Pourcel, Louise, Rain, & Tiollais, 1980; Shafritz, Shouval, Sherman, Hadziyannis, & Kew, 1981). HBV integration leads to the elevated expression of several cellular cancer-related genes, such as TERT, mixed-lineage leukemia 4 (MLL4) (MLL4 is a part of the ASC-2 complex implicated in the p53 tumor suppressor pathway (J. Lee et al., 2009)) and CCNE1 (encoding cyclin E1) (Sung et al., 2012). HBV integration is also associated with early onset of HCC and poor outcomes (Sung et al., 2012), and integrated HBV sequences encoding the HBx and/or truncated envelope pre-S2/S proteins are found in a large percentage of HCC (Tsai & Chung, 2010). Integration of the HBx sequence into host DNA in HCC promotes genetic instability through mechanisms that include the inactivation of the UV-damage DNA binding protein, so as to interfere with nucleotide excision repair, repression of p53-mediated gene transcription (S. G. Lee & Rho, 2000) and inactivation of p53-dependent apoptosis, cell cycle regulation, DNA repair and tumor suppression

(Kremsdorf, Soussan, Paterlini-Brechot, & Brechot, 2006). In addition, HBx transactivates several signaling pathways connected to carcinogenesis, including those mediated by protein kinase C, JAK/STAT and PI3K. (Feitelson & Lee, 2007; Feitelson et al., 2009). HBx also upregulates TGF-β expression in HCC tissue and is thought to contribute to carcinogenesis through this mechanism, as well (Yoo et al., 1996). TGF-β is a cytokine that inhibits hepatocyte proliferation during liver regeneration (Braun et al., 1988; Fausto & Mead, 1989; Nakamura et al., 1985) and stimulates extracellular matrix protein production by hepatocytes during liver cirrhosis (Czaja et al., 1989; Nakatsukasa et al., 1990). Deletion of the preS2 region of the S2/S protein during HBV integration leads to a truncated envelope pre-S2/S protein that is frequently found in HCC samples (Tai, Suk, Gerlich, Neurath, & Shih, 2002) (see Figure 1). This truncated pre-S2/S product increases malignant transformation by transactivating several cellular genes, including c-myc, c-fos and c-Ha-ras (Luber et al., 1996; Schluter, Meyer, Hofschneider, Koshy, & Caselmann, 1994). Interestingly, the pre-S mutant large surface antigens can be retained in the endoplasmic reticulum and, thus, escape detection by the immune system. Moreover, this protein can initiate ER stress and thereby induce oxidative DNA damage and genomic instability (H. C. Wang, Huang, Lai, & Su, 2006). The pre-S mutant also can upregulate COX-2 and cyclin A to induce cell-cycle progression and proliferation of hepatocytes (H. C. Wang et al., 2006). Overall, the process of HBV integration induces additional genetic alterations, including chromosomal deletions, translocations, fusion of transcripts, amplification of DNA and generalized genomic instability (Bonilla Guerrero & Roberts, 2005; Feitelson & Lee, 2007), leading to overexpression of oncogenes, depletion of tumor suppressor genes and an altered microRNA profile (Feitelson & Lee, 2007).

The integration rate of HBV DNA into the host genome is significantly increased in the presence of DSBs (Bill & Summers, 2004). If DNA damage is induced by the addition of H_2O_2, which increases cellular levels of ROS, the frequency of HBV integration also increases significantly (Dandri et al., 2002). Moreover, inhibiting poly(ADP-ribosyl)ation by adding the ADP-ribosylation inhibitor, 3-aminobenzamide, also leads to a significant increase in integration (Dandri et al., 2002). Poly(ADP-ribose) polymerase-1 (PARP-1) is an enzyme that is stimulated by DNA strand breaks caused by oxidative stress and other types of DNA damage and responds by carrying out poly(ADP-ribosyl)ation. Poly(ADP-ribosyl)ation is involved in DNA damage repair processes, such as base excision repair and suppression of genetic recombination (Burkle, 2001; Meyer, Muller, Beneke, Kupper, & Burkle, 2000; Trucco, Oliver, de Murcia, & Menissier-de Murcia, 1998; S. Yoshida & Simbulan, 1994).

Integration of HBV into the human genome can occur near or within fragile sites in genes that regulate cell signaling, proliferation and viability (Murakami et al., 2005). Common targets of integration include genes for human cyclin A2 (J. Wang et al., 1992), the PDGF receptor, calcium signaling-related genes, mixed lineage leukemia encoding genes, 60S ribosomal protein genes (Murakami et al., 2005), human telomerase reverse transcriptase (hTERT) (Horikawa & Barrett, 2001) and the retinoic acid receptor β (Yaginuma et al., 1987). The data produced by Next Generation Sequencing (NGS) now allows researchers to not only determine the sites of integration, but also to identify the mutations and to specify DNA damage types that contribute to carcinogenesis (Barzon, Lavezzo, Militello, Toppo, & Palu, 2011; S. Li & Mao, 2013). NGS studies have shown that HBV integrations into TERT, MLL4, CCNE1 and ANGPT1 (encoding angiopoietin

1) (Fujimoto et al., 2012; Jiang et al., 2012; Sung et al., 2012) can and do occur. NGS has also identified frequent mutations in CTNNB1 (encoding β-catenin), IRF2 (encoding interferon regulatory factor 2), TP53, ARID2 (subunit of the polybromo- and BRG1-associated factor chromatin remodeling complex (Yan et al., 2005); functioning as a tumor suppressor gene (M. Li et al., 2011)) and ARID1A (encoding a component of the SWI/SNF chromatin remodeling complex) (Guichard et al., 2012; J. Huang et al., 2012; M. Li et al., 2011). These mutations can be associated with disease etiology. For example, ARID2 mutations are significantly enriched in HCV-associated HCC (M. Li et al., 2011), and ARID1A may be crucial in HCC invasion and metastasis (J. Huang et al., 2012). IRF2 mutations have been associated with hyperploidy and high chromosomal instability (Guichard et al., 2012), while TP53 inactivating mutations result in an altered p53 pathway (Guichard et al., 2012). Interestingly, CTNNB1 mutations may define a homogenous subtype of HCC not related to HBV infection (Guichard et al., 2012).

Human Papillomaviruses (HPVs)

HPVs are a group of circular, double-stranded DNA viruses that infect epithelial cells. More than 100 different genotypes of HPV (Calleja-Macias et al., 2005) have been described; of these, a subset infects the anogenital area, and within this subset, the individual types are classified as either high risk or low risk. High-risk HPVs can cause cancerous lesions, while low-risk HPVs do not (Ault, 2007). An important difference between high- and low-risk HPVs is that high-risk HPVs show a greater tendency to integrate into the host genome, thereby causing high-grade lesions and cancer, while low-risk types are preferentially maintained as extrachromosomal circular episomes (Arends, Buckley, & Wells, 1998; Scheurer, Tortolero-Luna, & Adler-Storthz, 2005).

HPV is known to cause virtually all cases of cervical cancer, which is the second most common cancer in women worldwide and the fourth most common cause of cancer death in women worldwide (Bernard W. Stewart, 2014; Parkin & Bray, 2006). More than 270,000 women die from cervical cancer each year, and according to 2013 data from the WHO, the developing world accounts for more than 85% of these cases. More than 90% of premalignant and malignant squamous lesions in the uterine cervix are HPV DNA positive (Clifford et al., 2005; zur Hausen, 1991). Moreover, HPV16, HPV18, HPV31 and HPV33 account for 90% of all cases of cervical cancer. Among these high-risk HPVs, HPV type 16 is the most prevalent type and by itself accounts for more than 50% of all cases of cervical cancer (Liu, Tergaonkar, Krishna, & Androphy, 1999; Lowy, Kirnbauer, & Schiller, 1994). High-risk HPV infection is also associated with several other anogenital and oropharyngeal cancers. For example, it is thought to be responsible for more than 90% of anal cancers, 70% of vaginal and vulvar cancers, 60% of penile cancers and 63% of oropharyngeal cancers ("Human papillomavirus-associated cancers - United States, 2004-2008," 2012).

The majority of HPV infections are spontaneously cleared by human cells (Song et al., 2006; Vinokurova et al., 2008). Cervical intraepithelial neoplasia (CIN) 1 lesions develop occasionally, but most of these lesions will be spontaneously cleared and fail to progress (Ostor, 1993). However, some HPV-infected women can be co-infected by other viruses or bacteria, then develop cervical inflammation, as noted above. The cellular proliferative and anti-apoptotic effects of inflammation, combined with low-level expression of the E6 and E7 oncogenes encoded by the episomal HPV, contribute to CIN1 to CIN2 progression (Williams et al., 2011). At the CIN2 stage, the generation of

ROS and RNS may create DSBs in both the viral and host DNA. This can then allow HPV to integrate into the human genome, thereby enabling overexpression of the E6 and E7 oncogenes, which then facilitate the transition to CIN3 and, sometimes, invasive carcinoma.

The oncogenes encoded by HPV play crucial roles in carcinogenesis. Typically, the levels of E6 and E7 oncogene expression from episomal HPV16 are low. *In vitro*, high-level expression of HPV oncogenes from integrated HPV forms is generally preceded by the loss of episomal HPV (Peitsaro, Hietanen, Johansson, Lakkala, & Syrjanen, 2002; M. R. Pett et al., 2006). HPV-16 integration often disrupts the E2 gene (the E2 ORF has been identified as a preferential site of integration), resulting in significantly increased expression of the E6 and E7 oncoproteins due to loss of negative feedback control by the viral regulatory E2 protein (M. Pett & Coleman, 2007; von Knebel Doeberitz, 2002) (see Figure 1). Consistent with this model, transcriptional activity of integrated HPV-16 DNA can be suppressed by E2 proteins from the episomal form (Bechtold, Beard, & Raj, 2003; Hafner et al., 2008; Herdman et al., 2006; M. Pett & Coleman, 2007; M. R. Pett et al., 2006).

Elevated expression of HPV oncogenes can also be achieved by the presence of an increased number of episomal HPV-16 copies. A high episomal HPV-16 load, combined with an absence of HPV-16 integration, is frequently observed in CIN, while a high proportion of invasive cancers contain integrated HPV-16 forms, suggesting that integration is an important factor in progression from high-grade lesions to invasive cancer and may be a potential marker for CIN progression risk assessment (Guo et al., 2007; Hopman et al., 2004).

Accumulating evidence suggests that among those factors that can increase the risk of HPV integration are those factors that generate excessive amounts of ROS and RNS. Nitrative and oxidative DNA damage were found in high-risk HPV infections, especially nitric oxide synthase-dependent DNA damage, which is believed to play a critical role in inflammation-mediated cervical carcinogenesis (Hiraku et al., 2007). Exposure to high and sustained levels of nitric oxide (NO) will increase DNA damage and mutation frequencies, supporting the idea that NO is a cofactor with infection in cervical carcinogenesis (Wei, Gravitt, Song, Maldonado, & Ozbun, 2009). Furthermore, ROS and RNS have the potential to create DNA strand breaks (Wei et al., 2009; Ziech, Franco, Pappa, & Panayiotidis, 2011), thereby increasing HPV-DNA integration into cellular chromatin. As discussed above, these factors may be virus mediated (*i.e.*, E6* expression) (Williams et al., 2014a), mediated through infection or co-infection (inflammation) or exogenously derived (Williams et al., 2011). Our laboratory has demonstrated that chronic oxidative stress induced by E6* expression is able to increase DNA damage (Williams et al., 2014a), and it has also been shown by other laboratories that the frequency with which foreign DNA constructs containing antibiotic resistance markers integrate into genomic DNA is increased by the induction of DSBs, either by HPV16 E6 and E7 expression or by spontaneous breakage at fragile sites in specific cancer cell lines (Kessis, Connolly, Hedrick, & Cho, 1996; Matzner, Savelyeva, & Schwab, 2003; T. Yu et al., 2005). Therefore, the rate of HPV integration seems to correlate with the level of DNA damage.

A number of pathways are affected by E6 and E7 expression (zur Hausen, 2000). As mentioned earlier, E6 mediates the rapid degradation of p53 and activates hTERT,

while E7 inactivates pRB. In addition, E6 binds to IRF-3 and inhibits its transcriptional activity, which may provide HPV with the ability to circumvent the normal antiviral response (Ronco, Karpova, Vidal, & Howley, 1998). E7 sequences mediate the activation of cyclin E, followed by the activation of cyclin A, which is required for transformation (Zerfass et al., 1995). In addition, the cooperative interactions between the E6 and E7 proteins leads to cellular immortalization (Band, Zajchowski, Kulesa, & Sager, 1990; Munger & Phelps, 1993), likely through a combination of specific mechanisms, such as those noted above. In summary, the elevated expression of oncoproteins from integrated forms of HPV deregulates cellular proliferation, blocks apoptosis and increases genomic instability, all of which contribute to cellular transformation (see Figure 1).

HPV integration sites are distributed randomly throughout the host genome, without a single region predominating (Wentzensen, Vinokurova, & von Knebel Doeberitz, 2004). However, 38% of 192 integrants were found in known common fragile sites (CFSs), and there was no evidence to suggest targeted disruption or functional alteration of critical cellular genes by the integrated viral sequences (Wentzensen et al., 2004). However, some studies have demonstrated that high-risk HPV integration has occurred within or adjacent to known oncogenes, most commonly within intronic sequences (Ferber et al., 2003; Thorland, Myers, Gostout, & Smith, 2003; Wentzensen et al., 2002; Wentzensen et al., 2004). For example, the region of the MYC gene at chromosomal band 8q24 is a frequently observed integration site in HPV18-positive cervical cancer (Ferber et al., 2003; Peter et al., 2006; Wentzensen et al., 2002). Recently developed NGS-based methods now provide a very efficient method to map HPV integration sites. One NGS study, for example, showed that the 3'-breakpoints of integrated HPV16 DNA

distribute preferentially within the early region E1-PAE segment in HPV 16. This indicates the importance of deregulated viral oncogene expression for carcinogenesis (Xu et al., 2013). Interestingly, about half of the mapped HPV16 integration sites directly target human cellular genes (Xu et al., 2013). These studies suggest that the insertional mutagenesis of the host genome may play a role in at least some cervical cancers (M. Pett & Coleman, 2007). However, many publications using NGS focus on identifying genotype and determining HPV load, rather than on identifying sites of integration (Conway et al., 2012; Meiring et al., 2012). Further studies are needed to identify integration sites, genomic mutation and DNA damage.

Merkel Cell Polyomavirus (MCV)

MCV is a double-stranded DNA polyomavirus, shown to be associated with Merkel cell carcinoma (MCC) through the use of NGS in 2008 (Feng et al., 2008). These tumors display MCV DNA in an integrated form within the tumor cell genomes in a clonal pattern, suggesting that MCV infection and integration contribute to clonal expansion of the tumor cells (Feng et al., 2008; Sastre-Garau et al., 2009) (see Figure 1). The MCV genome encodes multiple splice variants of a tumor (T) antigen protein complex that targets several tumor suppressor proteins, such as pRB and p53 (Shuda et al., 2008). One of these splice variants, the large tumor antigen, is mutated in MCV-positive MCC tumors cells, and this selective mutation affects the cellular DNA damage response to prevent auto-activation of integrated virus replication, disrupt host genomic integrity and inhibit cellular proliferation (Demetriou et al., 2012; J. Li et al., 2013; Shuda et al., 2008). Several features of this virus, such as the frequent and selective association of MCC with MCV, integration of MCV into the host genome, the recurrent

pattern of conserved viral DNA sequences and the constant expression of viral oncoproteins are very similar to those seen in high-risk HPV-induced cervical cancer [55]. However, the exact role of integration in MCC carcinogenesis requires further study.

Epstein-Barr Virus (EBV)

EBV is a double-stranded DNA herpesvirus that is primarily associated with Burkitt's lymphoma, nasopharyngeal carcinoma and several lymphoproliferative disorders (Epstein et al., 1964). Burkitt's lymphoma appears in three main clinical variants—the endemic, sporadic and immunodeficiency-associated variants. EBV is detected in 96% of cases of endemic variant Burkitt's lymphoma involving the jaw, which is the most common malignancy of children in certain areas of central Africa (zur Hausen et al., 1970). In contrast, EBV is rarely associated with the sporadic variant of Burkitt's lymphoma, and the jaw is less commonly involved (Xing & Kieff, 2007). EBV-associated Burkitt's lymphoma is common in individuals lacking efficient T-cell function, such as AIDS patients or transplant recipients (Kieff. E, 2007).

All Burkitt's lymphomas have chromosomal translocations that place the MYC oncogene under the control of the Ig heavy chain or one of the light-chain loci, which induce MYC deregulation and contribute to the pathogenesis associated with Burkitt's lymphoma (Kovalchuk et al., 2000; Z. Li et al., 2003; Polack et al., 1996). EBV also displays transformative abilities, as EBV-encoded latent genes induce B-cell transformation into permanent, latently-infected lymphoblastoid cell lines (LCLs) *in vitro* by altering cellular gene transcription and activating important cell-signaling pathways (Young & Rickinson, 2004).

EBV usually persists in an episomal state with multiple copies of circular DNA. Integration of the EBV genome into that of the host is rare and is unlikely to contribute to most cases of Burkitt's lymphoma. Such integration has been only shown in several Burkitt's lymphoma cell lines, such as IB4, BL-36, BL-60 and BL-137 (Delecluse, Bartnizke, Hammerschmidt, Bullerdiek, & Bornkamm, 1993; Jox et al., 1997; Matsuo, Heller, Petti, O'Shiro, & Kieff, 1984; Popescu, Chen, Simpson, Solinas, & DiPaolo, 1993; Wolf et al., 1993). Integrated, episomal and linear copies of EBV DNA can coexist in Burkitt's lymphoma cells (Delecluse et al., 1993). EBV integration into fragile sites of the host chromosome is associated with partial deletion of the viral genome and generates a region of enhanced chromatin instability in the host cell genome (Jox et al., 1997). This genome instability can induce the loss of host genes, such as BACH2, which is a putative tumor suppressor gene, and this may contribute to lymphomagenesis (Takakuwa et al., 2004).

In addition to Burkitt's lymphoma, EBV is also associated with nasopharyngeal carcinoma (NPC). The undifferentiated form of NPC, classified by WHO as type III, shows the most consistent association with EBV worldwide, especially in particular areas of China and south-east Asia (M. C. Yu & Yuan, 2002). An association between EBV and WHO types I and II NPC has also been demonstrated (Pathmanathan et al., 1995). Evidence of the importance of EBV integration for the development NPC is not conclusive, and integration of EBV was demonstrated in only a subset of studies. For example, integrated EBV was found in four of 17 pathologically-diagnosed, EBV-positive NPC biopsy samples (Kripalani-Joshi & Law, 1994). Another study showed that EBV DNA

was integrated into chromosomes in the EBV-positive NPC cell lines, HSB4 and H2B17-7. However, the exact role of integration of EBV in NPC is not clear.

Prevention and Reduction of Risk Factors for Cancers

Knowledge of the etiology of virus-mediated carcinogenesis, the networking of pathways involved in the transition from infection to cancer and the risk factors associated with each type of cancer, all suggest prophylactic and therapeutic strategies that may reduce the risk of virus-mediated cancer. Prophylactic vaccination provides one such strategy; HPV vaccines can effectively reduce the incidence of HPV infections and are anticipated to correspondingly reduce the incidence of HPV-associated cancers and pre-cancerous lesions over the next several decades (Saslow et al., 2007). As discussed here, lifestyle choices also matter, and education on the risk factors associated with these lifestyle choices can be considered a prophylactic measure targeting cancer prevention. We also note factors that contribute to virus-mediated carcinogenesis, including integration of the viral genomes of HBV, HPV and MCV. Typically, cancer develops years to decades following the initial infection with HBV and HPV. This delay provides us with a unique opportunity for cancer interception, especially in cases where the tumorigenesis process requires virus integration. In these situations, approaches that would decrease the probability of integration are anticipated to reduce the number of cancer cases. Theoretically, therapeutic or dietary measures directed against oxidative stress could reduce oxidative stress in already infected cells (Garcia-Closas, Castellsague, Bosch, & Gonzalez, 2005) and thereby diminish the risk of viral integration. Dietary antioxidants can, in some cases, supplement the activity of endogenous antioxidants found in normal cells and fortify them against challenges posed by increased levels of ROS. In the case of

cervical cancer, studies have shown that several antioxidants, such as reduced glutathione, GPx, glutathione-S-transferase, SOD and antioxidant vitamins (vitamin E, vitamin C, lutein, beta-carotene, lycopene and zeaxanthin), are reduced in the circulation of cervical cancer patients (Y. T. Kim et al., 2004; Manju, Kalaivani Sailaja, & Nalini, 2002). In contrast, the concentration of the lipid peroxidation parameter, malondialdehyde, was significantly higher in women with CIN or invasive cervical cancer (Y. T. Kim et al., 2004). That may be due to the increased utilization of the antioxidants to scavenge oxidative stress-induced lipid peroxidation and their sequestration by tumor cells. A deficiency in antioxidant vitamins may, therefore, contribute to the increased prevalence of cervical cancer observed in women with a low socioeconomic status (Manju et al., 2002). Possible benefits from dietary antioxidant consumption, a highly researched topic for the last two decades, still remain debatable (Halliwell, 2007; Manda, 2009). In addition, clinical trials addressing the effect of antioxidant therapies on cancer have not yet provided a clear answer (Bjelakovic, Nikolova, Simonetti, & Gluud, 2004). Future development of therapies for the reduction of cellular oxidative stress must take into account the complexity of problems inherent in the regulation of redox balance.

CHAPTER TWO

CHRONIC OXIDATIVE STRESS INCREASES THE INTEGRATION FREQUENCY OF FOREIGN DNA AND HUMAN PAPILLOMAVIRUS 16 IN HUMAN KERATINOCYTES

Running title: Oxidative stress increases HPV integration frequency

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Chronic oxidative stress increases the integration frequency of foreign DNA and human papillomavirus 16 in human keratinocytes. American J for Cancer Res. 2016; In Press.

Abstract

Cervical cancer is the second most common cancer, and the fourth most common cause of cancer death in women worldwide. Nearly all of these cases are caused by high-risk HPVs (HR HPVs), of which HPV16 is the most prevalent type. In most cervical cancer specimens, HR HPVs are found integrated into the human genome, indicating that integration is a key event in cervical tumor development. An understanding of the mechanisms that promote integration may therefore represent a unique opportunity to intercept carcinogenesis. To begin identifying these mechanisms, we tested the hypothesis that chronic oxidative stress (OS) induced by virus- and environmentally-mediated factors can induce DNA damage, and thereby increase the frequency with which HPV integrates into the host genome. We found that virus-mediated factors are likely involved, as expression of E6*, a splice isoform of HPV16 E6, increased the levels of reactive oxygen species (ROS), caused oxidative DNA damage, and increased the frequency of plasmid DNA integration as assessed by colony formation assays. To assess the influence of environmentally induced chronic OS, we used L-Buthionine-sulfoximine (BSO) to lower the level of the intracellular antioxidant glutathione. Similar to our observations with E6*, glutathione depletion by BSO also increased ROS levels, caused oxidative DNA damage and increased the integration frequency of plasmid DNA. Finally, under conditions of chronic OS, we were able to induce and characterize a few independent events in which episomal HPV16 integrated into the host genome of cervical keratinocytes, using the APOT (Amplification of Papillomavirus Oncogene Transcripts) approach. Our results demonstrate the chain of events leading from induction of oxidative stress, to DNA damage, to viral integration, and ultimately to carcinogenesis.

Keywords

Cervical cancer, high-risk HPVs (HR HPVs), integration, oxidative stress (OS), reactive oxygen species (ROS), E6*, carcinogenesis.

Introduction

Cervical cancer is the second most common cancer in women, and the fourth most common cause of cancer-related death in women worldwide (Bernard W. Stewart, 2014; Parkin & Bray, 2006). Human papilloma virus (HPV) infection is well-established as the causative agent of cervical cancer (Ahn et al., 2003), and at least 85% of premalignant and 90% of malignant squamous lesions in the uterine cervix test HPV DNA positive (Smith et al., 2007). High-risk HPV such as HPV type 16 is the most prevalent type and accounts for more than 50% of all cases of cervical cancer (Lowy et al., 1994).

Over-expression of the HR E6 and E7 oncoproteins are considered responsible for most malignancies. This over-expression can be triggered by several mechanisms, including epigenetic modifications of the HPV genome (Szalmas & Konya, 2009). However, integration of the HPV genome into that of the host, with an accompanying loss of E2 and the resulting over-expression of E6 and E7, appears to be one of the most common oncogenic pathways (Baker et al., 1987). Following viral infection, the HPV genome is present in episomal form, with low levels of E6 and E7 oncoprotein expression due to the suppressive activity of E2 (M. Pett & Coleman, 2007). The level of oncogene expression grows with increase of virus load (Rosty et al., 2005) and following integrative events that disable E2-mediated suppression of E6 and E7 (M. Pett & Coleman, 2007). Clinical data support this sequence, as precancerous lesions CIN1 and CIN2 display episomal viral genomes (Cullen, Reid, Campion, & Lorincz, 1991), while rapid progression of CIN lesions is closely associated with integrated HPV16 (Peitsaro, Johansson, & Syrjanen, 2002) and is accompanied by increased levels of E6 and E7

expression. In 88% of specimens obtained from cervical cancers, HPV DNA is found integrated into host genomes (Klaes et al., 1999).

Integration requires linearization of the viral genome and breakage of the host genome, along with the ability to re-ligate the ends together. Therefore, agents that cause DNA breakage are predicted to increase integration frequency. Perhaps the most common agents of DNA damage are reactive oxygen radicals (ROS). Indeed, in the case of hepatitis B virus (HBV), oxidative stress (OS) has been shown to enhance viral integration by increasing DNA damage (Dandri et al., 2002; Petersen, Dandri, Burkle, Zhang, & Rogler, 1997); this integration then serves as a key step during HBV-initiated carcinogenesis (Cougot, Neuveut, & Buendia, 2005). OS can be generated both by the virus itself and through environmental agents. Virus infection alone has the potential to induce OS, as seen in cases such as HBV and Epstein-Barr virus (EBV) (Cerimele et al., 2005; Y. Chen, Williams, Filippova, Filippov, & Duerksen-Hughes, 2014; Hagen et al., 1994; Shimoda et al., 1994). In contrast to the situation seen with these two viruses, infection with HPV does not in itself seem to cause significant inflammation likely to lead to OS, suggesting that any connection between HPV and OS is likely mediated by specific proteins. Support for a connection between specific HPV proteins and integration, possibly mediated by increased OS, is provided by the observation that expression of HR HPV E6 and E7 can promote integration of a reporter plasmid (Kessis et al., 1996). Previous work from our laboratory demonstrated that at least one HPV-encoded protein, E6*, is able to increase ROS levels and DNA damage (Williams, Filippova, Filippov, Payne, & Duerksen-Hughes, 2014b). E6* is a splice variant of E6; interestingly, the full-length E6 protein does not affect ROS levels in the host cell

(Williams et al., 2014b). We also showed that an E6*-mediated increase in ROS is connected to a decrease in the expression of antioxidant enzymes such as superoxide dismutase (SOD2) and glutathione peroxidase (GPx ½) (Williams et al., 2014b).

In addition to such viral contributions to OS, environmental contributions are also likely. For example, epidemiological data link conditions known to cause oxidative stress and DNA damage, such as smoking and co-infection with the STD-associated pathogens *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, with increased incidence of HPV-mediated cancers (Castle & Giuliano, 2003; Castle et al., 2001; Deacon et al., 2000; Finan et al., 2006; Hawes & Kiviat, 2002; Plummer, Herrero, Franceschi, Meijer, Snijders, Bosch, de Sanjose, & Munoz, 2003; Williams et al., 2011), while corroborating data from the Ozbun lab demonstrated that tobacco exposure increased E6/E7 oncogene expression, DNA damage and mutation rates in cells carrying HPV episomes (Wei, Griego, Chu, & Ozbun, 2014), and that nitric oxide increases DNA damage in HPV positive cells (Wei et al., 2009).

However, the mechanistic link between OS and HPV DNA integration has not yet been examined, leading us to ask if we could induce HPV integration by generating OS-induced DNA damage. To investigate the relationship between chronic OS, DNA damage, and the rate at which episomal HPV DNA integrates into the cellular genome, we first performed a validation study using a U2OS cell line model and found that overexpression of E6* increased the rate of circular plasmid DNA integration, while manipulating the level of OS modified the integration frequency. We were also able to demonstrate the relationship between OS induction, DNA damage and increased plasmid integration in Normal Oral Keratinocytes (NOK cells). Finally, by treating Human

Cervical Keratinocytes (HCK, which normally carry the HPV episome) with the glutathionine-depleting agent L-Buthionine-sulfoximine (BSO), we were able to increase the rate at which the HPV episome integrated into the genomic DNA. In summary, we found that chronic OS, caused either by E6* overexpression or by BSO treatment, increased the levels of cellular DNA damage, leading to an increased frequency of integration for plasmid and episomal HPV16 DNA.

Materials and Methods

Reagents

L-Buthionine-sulfoximine (BSO) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solution, at a concentration of 100 mM, was prepared in phosphate-buffered saline (PBS) and kept at -20°C. Vitamin E and Resveratrol were purchased from Sigma-Aldrich (St. Louis, MO). Flag-agarose and anti-Flag-HRP antibodies were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

U2OS cells derived from a human osteosarcoma were obtained from the ATCC (Manassas, VA), and were cultured in McCoy's 5A medium (Invitrogen, Carlsbad, CA) that was supplemented to contain 10% fetal bovine serum (FBS) (Invitrogen). The U2OS cell line was authenticated by ATCC short tandem repeat profiling in November, 2015 and exhibited no evidence of cross-contamination with known ATCC cell lines.

Normal oral keratinocytes (NOKs), non-transformed cells immortalized by Human Telomerase Reverse Transcriptase (hTERT) that were kindly provided by Dr.

Karl Münger (Piboonniyom et al., 2003), were grown in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA).

Human cervical keratinocytes (HCK) containing episomal HPV16, , kindly provided by Dr. Aloysius J. Klingelhutz (Sprague et al., 2002), were grown in E-media (Wu et al., 1982).

All media were supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich, St. Louis, MO).

Transfections and Transductions

Transfections were performed following the protocol provided with the TransIT-2020 reagent kit (Mirus Bio, Madison, WI). Briefly, U2OS or NOK cells were transfected with plasmids encoding E6* (pE6*), E6 large (pE6 large) or the empty vector (pFlag). To normalize for transfection efficiency, pMetLuc was co-transfected, following the protocol provided with the TransIT-keratinocyte transfection reagent kit (Mirus Bio, Madison, WI). Expression of MetLuc was monitored in the media, and these values used for normalization.

Stable clones were obtained by transduction of retroviruses pLNCX, pLNCX-E6*, or pLNCX-E6 into NOK cells. Individual selected clones were analysed for protein expression by immunoblotting and/or real-time PCR as described previously (Williams et al., 2014b).

Clonegenic Assay

5×10^5 cells were transfected with pMetluc and incubated for 48 hours under cell culture conditions, followed by the addition of appropriate antibiotics (*G418* at a

concentration of 0.5 mg/ml for U2OS cells or *puromycin* at a concentration of 5 μg/ml for NOK cells) and selection for 2-3 weeks. After antibiotic selection, colonies were fixed using 10% formaldehyde for 30-45 min. After rinsing with water and drying overnight at room temperature, cells were stained with 1% crystal violet for 45-60 min. Dishes were rinsed with water and left to dry overnight at room temperature. Colonies were counted the following day.

Immunoprecipitation

10^6 cells from each assessed U2OS preparation, transiently transfected with plasmids encoding E6* (pE6*), E6 large (pE6L) or vector (pFlag), were lysed using lysis buffer (Filippova, Song, Connolly, Dermody, & Duerksen-Hughes, 2002). Flag-tagged proteins were then precipitated using Flag-agarose, and bound proteins were subjected to SDS-PAGE. Following transfer to a polyvinylidene difluoride (PVDF) membrane, the Flag-E6* and Flag-E6 proteins were detected by immunoblotting using anti-Flag-HRP antibodies (Williams et al., 2014b).

Flow Cytometry Assessment of ROS

Cellular levels of hydrogen peroxide and hydroxyl and peroxyl radicals (H_2O_2, OH^-, and ROO^-) were measured following staining with 5-(and-6)- carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (Invitrogen, Carlsbad, CA), while cellular levels of superoxide ($O2^-$) were measured following staining with dihydroethidium (DHE) (Invitrogen, Carlsbad, CA) (Peshavariya, Dusting, & Selemidis, 2007). Stock solutions (20 mM) of the dyes were diluted with culture medium and added to cells to a final concentration of 5 μM DCFDA or 5 μM DHE. After incubating at 37°C in the dark

for 30 min, cells were trypsinized, washed, and collected using PBS. The DCFDA and DHE signals were detected in the FL-1 (530 nm) and FL-2 channels (650 nm) respectively with a Becton, Dickinson FACS Calibur flow cytometer (BD, Franklin Lakes, NJ). A total of 10,000 events were measured per sample. Data were collected and analysed using CellQuest Pro and FlowJo software (Williams et al., 2014b).

Glutathione Levels

To assess the level of intracellular glutathione, cells were seeded into 96-well cell culture plates and allowed to grow for 24 hours under cell culture conditions. Cells were then treated with the BSO for 48 hours. Intracellular glutathione levels were measured using the bioluminescent Promega GSH-Glo™ glutathione assay kit according to the manufacturer's protocol (Promega, Madison, WI). Luminescence was detected using a MicroLumat Plus LB 96V bioluminometer (Berthold Technologies, Oak Ridge, TN).

Comet Assessment of DNA Damage

The comet assay was performed following the protocol provided with the Trevigen kit (Trevigen, Gaithersburg, MD). Briefly, cells were plated in agarose and spread over the sample area on slides. Alkaline electrophoresis using 21 V for 40 min was then performed. Nuclei were stained with SYBR gold (Invitrogen, Carlsbad, CA) and viewed by fluorescence microscopy. 100 DNA tails were photographed and analysed for each sample, and the length of each tail was measured from the center of the comet to the end of the tail using ImageJ software. Each cell was categorized as having tail lengths in one of four classes (0 to 50, undamaged; 50 to 100, minimum damage; 100 to 150, medium damage; >150, maximum damage) reflecting the severity of DNA damage. Tail

lengths are known to increase proportionately with DNA damage (Williams et al., 2014b).

APOT (Amplification of Papillomavirus Oncogene Transcripts)

The APOT assay was applied to detect and distinguish episomal- and integration-derived HPV transcripts. RNA from 5×10^6 cells was isolated using a Direct-zol RNA MiniPrep kit (Zymo research, Irvine, CA) as recommended by the supplier. Total RNA (1 mg) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem by Life Technologies, Grand Island, NY), using an oligo$(dT)_{17}$-primer coupled to a linker sequence [$(dT)_{17}$-p3, 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'] and 1 µl of MultiScribe reverse transcriptase for 10 min at 25°C, then for 120 min at 37°C in a final volume of 20 µl.

APOT was performed as described previously by Klaes *et al* (Klaes et al., 1999). Briefly, cDNAs were amplified by the first PCR round using HPV E7-specific oligonucleotides [p1 specific for HPV16 (5'-CGGACAGAGCCCATTACAAT-3') as forward primers and p3 (5'-GACTCGAGTCGACATCG-3') as the reverse primer] in a 50 µl reaction mixture. The second PCR used forward primers p2 specific for HPV16 (5'-CTTTTTGTTGCAAGTGTGACTCTACG-3') and $(dT)_{17}$-p3 as the reverse primer in a total volume of 50 µl reaction mixture.

Amplification products were visualized by 1% agarose gel electrophoresis, and amplicons of sizes other than the episome-derived transcript (1050 bp) were subjected to further analysis (Klaes et al., 1999). PCR bands were extracted and cloned into pTOPO using the TOPO cloning reaction kit (Life Technologies, Grand Island, NY) and

sequenced. The sequences obtained were compared to those within the HPV16 and human genomes to identify similar sequences, using National Center for Biotechnology Information (NCBI) Blast as well as UCSC Genome Bioinformatics Blat tools.

Quantitative Reverse Transcription PCR (qRT-PCR)

Reverse transcription was performed as described above (APOT). Quantitative real-time PCR was conducted to measure the levels of the E6 isoforms using primers designed as described previously by Hafner et al. (Hafner et al., 2008), along with an Absolute QPCR SYBR Green Kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). The observed E6 isoform concentrations were normalized using the level of phosphoglycerokinase (PGK) expression.

Results

E6, but not E6, Increased the Level of OS and the Integration Frequency of Plasmid DNA into U2OS Cells*

Previously, we demonstrated that overexpression of E6*, but not of the full-length E6 isoform, caused increased cellular levels of ROS and higher levels of oxidative DNA damage (Williams et al., 2014b). To investigate whether overexpression of E6* also increases the rate at which circular plasmid DNA integrates into the human genome, we performed a clonogenic assay. U2OS cells were transfected with plasmids encoding one of the HPV 16 E6 isoforms, E6* (pE6*) or the full-length E6 (pE6L), or with the empty vector (pFlag), along with a plasmid coding for *G418* resistance. To normalize for transfection efficiency, the pMetLuc plasmid was co-transfected, and the expression of *Metridia* luciferase was monitored in the media. After *G418* selection, the remaining colonies were stained with crystal violet and counted (Figure 2A). The normalized number of colonies is presented in Figure 2B. These data show that the number of colonies was higher when cells were transfected with pE6* than when transfected with the vector or the pE6 large plasmids (Figure 2A and B). Immunoprecipitation followed by immunoblot analysis showed that E6* and E6 large were expressed in the corresponding cells (Figure 2C), and ROS levels following transient transfection with these plasmids, as estimated by fluorescence following DCFDA staining, were higher in cells transfected with E6* when compared to those transfected with pFlag or pE6L (Figure 2D). Overall, these results demonstrate that the short variant of the HR HPV E6 oncoprotein, E6*, but not the full-length variant (E6L), induces an increase in ROS and promotes the integration of plasmid DNA.

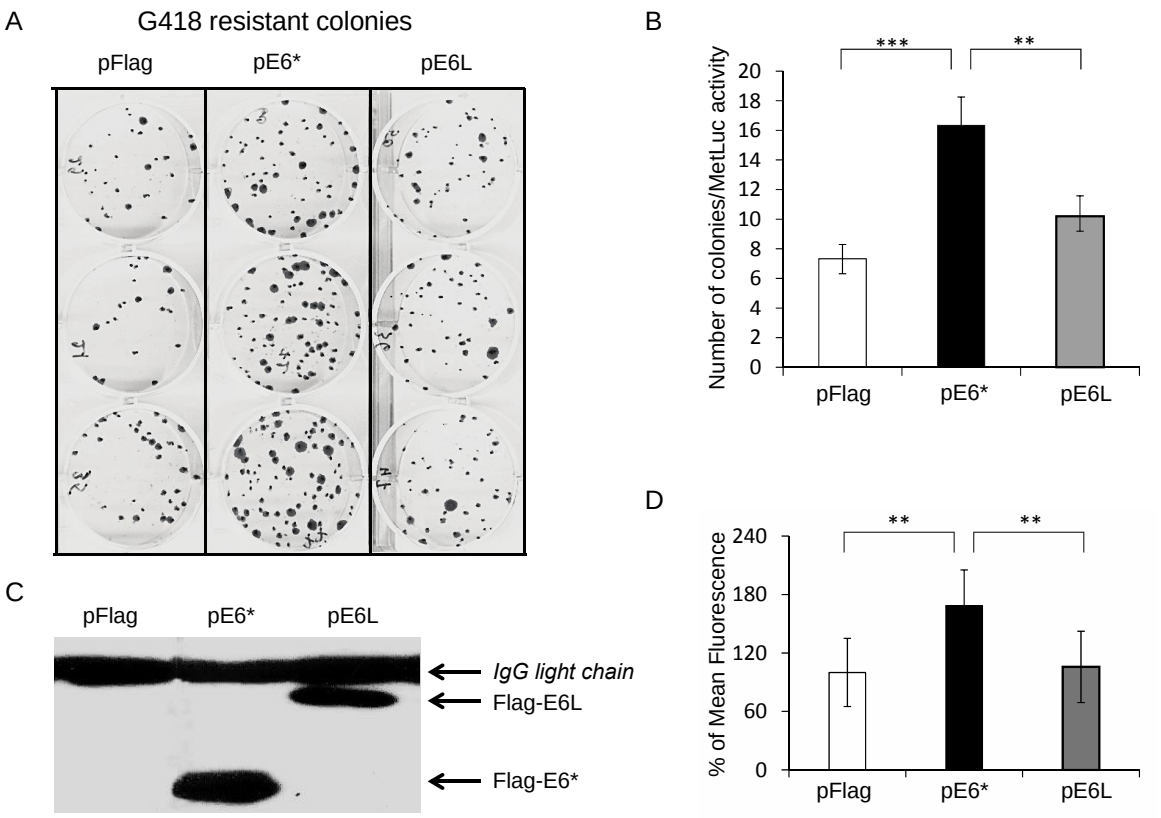


Figure 2. E6*, but not E6, increased the level of OS and the integration frequency of plasmid DNA into U2OS cells. **(A)** and **(B)** 10^6 U2OS cells per well were co-transfected with plasmids encoding E6* (pE6*), E6 (pE6L) or vector (pFlag) together with the pMetLuc *puro* plasmid. To normalize for transfection efficiency, the media was collected 48 h post-transfection and the expression of secreted MetLuc (BD Clontech) was measured. Selection of cells resistant to *G418* (0.5 mg/ml) was performed for 3 weeks and the resulting colonies were stained with crystal violet. Colonies were counted after crystal violet staining **(A)** and normalized for transfection efficiency **(B)**. **(C)** Immunoprecipitation followed by immunoblot shows expression of Flag-E6* and Flag-E6L proteins after transient transfection. **(D)** 10^5 cells per well of U2OS cells were transfected with plasmids encoding E6* (pE6*), E6 (pE6L) or vector (pFlag), and the level of cellular ROS level was analysed 48 h post-transfection by fluorescence using a fluorescence plate reader following DCFDA staining. Triplicate measurements of mean fluorescence intensity of DCFDA were performed to generate the bar graphs of % mean fluorescence, setting the pFlag mean fluorescence at 100%.

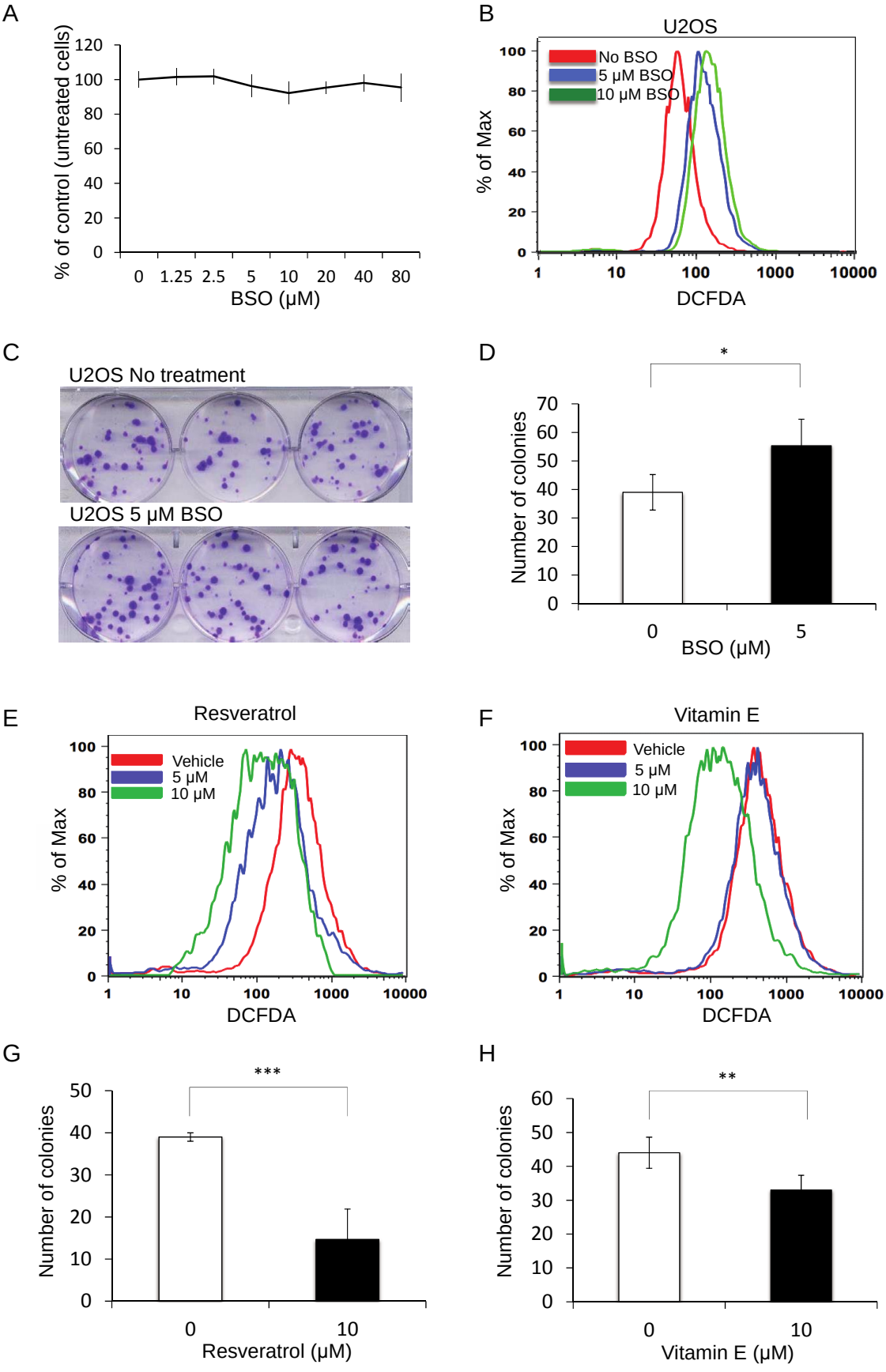
Increasing ROS Results in Increased Integration Frequency, while Decreasing ROS Results in Decreased Integration Frequency

To examine the impact of OS on integration frequency, we increased or decreased the levels of ROS using agents known to affect cellular levels of these reactants. To carry out this set of experiments, we needed an agent that would induce chronic OS in our cells by modulating ROS levels without significantly affecting cell viability. We found that L-Buthionine-sulfoximine (BSO), which decreases the level of glutathione in cells through inhibition of gamma glutamyl cysteine synthetase (gamma GCS), the enzyme that catalyzes the first step of glutathione synthesis (Armstrong et al., 2002), was suitable for our needs. Application of BSO to U2OS cells at doses from 1.25 μM to 80 μM did not significantly affect cell viability, as assessed by crystal violet staining (Figure 3A), while treatment with 5 and 10 μM of BSO for 48 hours led to elevation of ROS levels, as assessed by flow cytometry following DCFDA staining (Fig, 3B).

To determine whether chronic OS induced by BSO treatment increases the integration frequency of plasmid DNA in U2OS, we performed a clonogenic analysis of U2OS cells transfected with the pcDNA3 plasmid. After transfection, cells were plated onto a 6-well plate, and 3 wells were treated with 5 μM BSO for 5 days while the remaining 3 wells were used as controls. Following *G418* selection, the colonies were stained with crystal violet and counted (Figure 3C). The normalized number of colonies is presented in Figure 3D. These results demonstrate that chronic OS induced by BSO promotes the integration of plasmid DNA into U2OS cells.

To assess the effect of decreased ROS levels on integration frequency, cells were treated with the known antioxidants resveratrol (Leonard et al., 2003) and vitamin E

(Herrera & Barbas, 2001), and integration frequency was estimated using the clonogenic assay as described above. As expected, we found that ROS levels were significantly decreased after treatment with 5 μM and 10 μM resveratrol (Figure 3E) or with 10 μM Vitamin E (Figure 3F) for 48 hours as assessed by flow cytometry following DCFDA staining. After transfection with the pcDNA3 plasmid carrying the *G418*-resistant gene, cells were plated onto a 6-well plate, and 3 wells were treated with 10 μM resveratrol for 48 hours while the remaining 3 wells were left untreated. Following *G418* selection, the colonies were stained with crystal violet and counted (Figure 3G). A similar experiment was performed with U2OS cells treated with 10 μM Vitamin E (Figure 3H). These results clearly demonstrate that antioxidants that decreased ROS levels also caused a reduction in the rate at which circular plasmid DNA integrated into U2OS cells.


A
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10 μM BSO
% of Max
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Figure 3. Increasing ROS results in increased integration frequency, while decreasing ROS results in decreased integration frequency. **(A)** U2OS cells (10^4 cells per well) were plated onto a 96 -well plate, and cells were treated with BSO in triplicate at the indicated concentrations for 48 hours. Cell viability was estimated using the crystal violet assay. Cell viability without BSO treatment was set at 100%. **(B)** The level of ROS in U2OS cells treated with the indicated concentrations of BSO for 48 h was estimated by flow cytometry following DCFDA staining. **(C)** 1.5 x 10^5 cells per well of U2OS were transfected with the pcDNA3 plasmid, then plated onto a 6 well plate. After 24 hrs, 3 wells were treated with 5 μM of BSO for 5 days. Selection of cells resistant to *G418* (0.5 mg/ml) was performed for approximately 2 weeks, following which the colonies produced were stained with crystal violet. Colony numbers are presented in the bar graph **(D). (E)** and **(F)** The level of ROS in U2OS cells treated with the indicated concentrations of resveratrol **(E)** and vitamin E **(F)** for 48 h was estimated by flow cytometry following DCFDA staining. **(G)** and **(H)** 1.5 x 10^5 cells per well of U2OS were transfected with the pcDNA3 plasmid, then plated onto a 6 well plate. After 24 hrs, 3 wells were treated with 10 μM of resveratrol (G) or 10μM of Vitamin E (H) for 48 h. Selection of cells resistant to *G418* (0.5 mg/ml) was performed for approximately 1 week, and the colonies produced were stained with crystal violet. Colony numbers are presented in the corresponding bar graphs **(G)** and **(H)**. Error bars represent the standard deviation, * represents a 0.95 level of confidence, ** represents a 0.99 level of confidence, and *** represents a 0.999 level of confidence.

OS, Induced by either Overexpression of E6 or by Glutathione Depletion, Increases DNA Damage and the Frequency of Plasmid DNA Integration into NOK Cells*

To determine whether an increase of ROS in keratinocytes, the natural target cells for HPV infection, also results in an increased rate of circular DNA integration, we employed Normal Oral Keratinocytes (NOK), a cell line which was immortalized using hTERT (gift of Karl Münger) (Piboonniyom et al., 2003). NOK cells stably expressing either E6* alone, E6, or the empty vector control were obtained as described previously following transduction of the retroviruses pLNCX-E6*, pLNCX-E6 or pLNCX, respectively (Williams et al., 2014b). We first compared the level of E6* expression in NOK-derived pE6*, pE6L and pLNCX cells with that seen in CaSki and SiHa cells, where E6* is expressed from integrated HPV16 (Meissner, 1999), and with the level of E6* expressed in human cervical keratinocytes (HCK) using qRT-PCR. The level of E6* expression in NOK pE6* cells was approximately 2 times higher than that observed in HCK and CaSki cells, suggesting that the level of E6* expression from the estimated 50-100 copies of the HPV genome in HCK cells is comparable to that seen from the integrated HPV in CaSki cells, and that expression of E6* from the E6*-expressing NOK cells is well within a two-fold range of levels seen physiologically (Figure 4A).

NOK cells expressing E6* are characterized by higher levels of ROS (Figure 4B) and DNA damage (Williams et al., 2014b) than seen in NOK pLNCX or NOK E6L cells. To determine whether the plasmid integration rate was also higher in these cells, we transfected these pLNCX-E6*, pLNCX-E6 and pLNCX cells with the plasmid pMetLuc *puro*. To normalize for transfection efficiency, expression of secreted luciferase in the media was monitored. After *puromycin* selection, the number of colonies was counted

after crystal violet staining (Figure 4C). This clonogenic assay revealed that NOK cells expressing E6* produced a higher number of colonies, as compared to NOK cells expressing either E6 or those transfected with the empty vector. These results demonstrate that overexpression of E6* results in an increase in ROS along with enhanced DNA integration into keratinocytes, similar to our observations in the U2OS model system.

To assess the impact of external, OS-inducing agents on DNA damage and integration in NOK cells, we again employed BSO. We found that BSO at concentrations ranging from 5 μM to 20 μM does not affect cell viability, as assessed by crystal violet staining (Figure 4D). We also measured glutathione levels in these NOK cells before and after BSO treatment for 48 hours at several concentrations (5, 10, 20, and 40 μM) using the glutathione assay (Promega, Madison, WI). Figure 4E shows that BSO does, indeed, significantly reduce the level of glutathione in treated cells as compared to that seen in untreated cells. Treatment with BSO for 48 hours at concentrations of 12 and 100 μM in NOK cells increased ROS levels significantly (Figure 4F), as assessed by flow cytometry following DCFDA staining. Together, these results show that BSO is capable of inducing chronic cellular OS by decreasing levels of the antioxidant glutathione, without significantly affecting cell viability.

Next, we asked whether chronic OS induced by long-term BSO treatment would also increase DNA damage. NOK cells were treated with BSO for 9 days at a concentration of 5 μM, and DNA damage was estimated using the comet assay. The comet assay detects DNA strand breaks by measuring the distance DNA migrates out of cells (tail lengths), which corresponds to the severity of DNA damage (Collins, 2004).

The results of the comet assay (Figure 4G) demonstrate that the number of cells with longer tail lengths is increased in BSO-treated cells, as compared to an untreated control group. Representative cells with and without BSO treatment are shown in Figure 4H.

To determine whether chronic OS induced by BSO treatment also increases the integration frequency of plasmid DNA in keratinocytes, we performed a clonogenic analysis of NOK cells transfected with the pMetLuc *puro* plasmid. After transfection, cells were plated onto a 6-well plate, and 3 wells were treated with BSO for 48 hours while the remaining 3 were used as controls. Following *puromycin* selection of treated and untreated cells, the colonies were stained with crystal violet. No *puromycin* resistant clones were observed in wells without BSO treatment, while numerous stable clones were noted in wells treated with BSO (Figure 4I). These results clearly demonstrate that chronic OS induced by BSO promotes the integration of plasmid DNA.

The chronic OS that increased integration rates in NOK cells was induced by either overexpression of E6* (Figure 4C) or BSO treatment (Figure 4I). We compared the cellular levels of ROS resulting from E6 and E6* expression with those observed following treatment with 10 μM BSO. The results shown in Figure 4B demonstrate that exogenous application of the glutathione depleting agent BSO and expression of E6* in NOK cells lead to comparable levels of OS, suggesting that both environmental and viral factors may make significant contributions to the overall oxidative status.

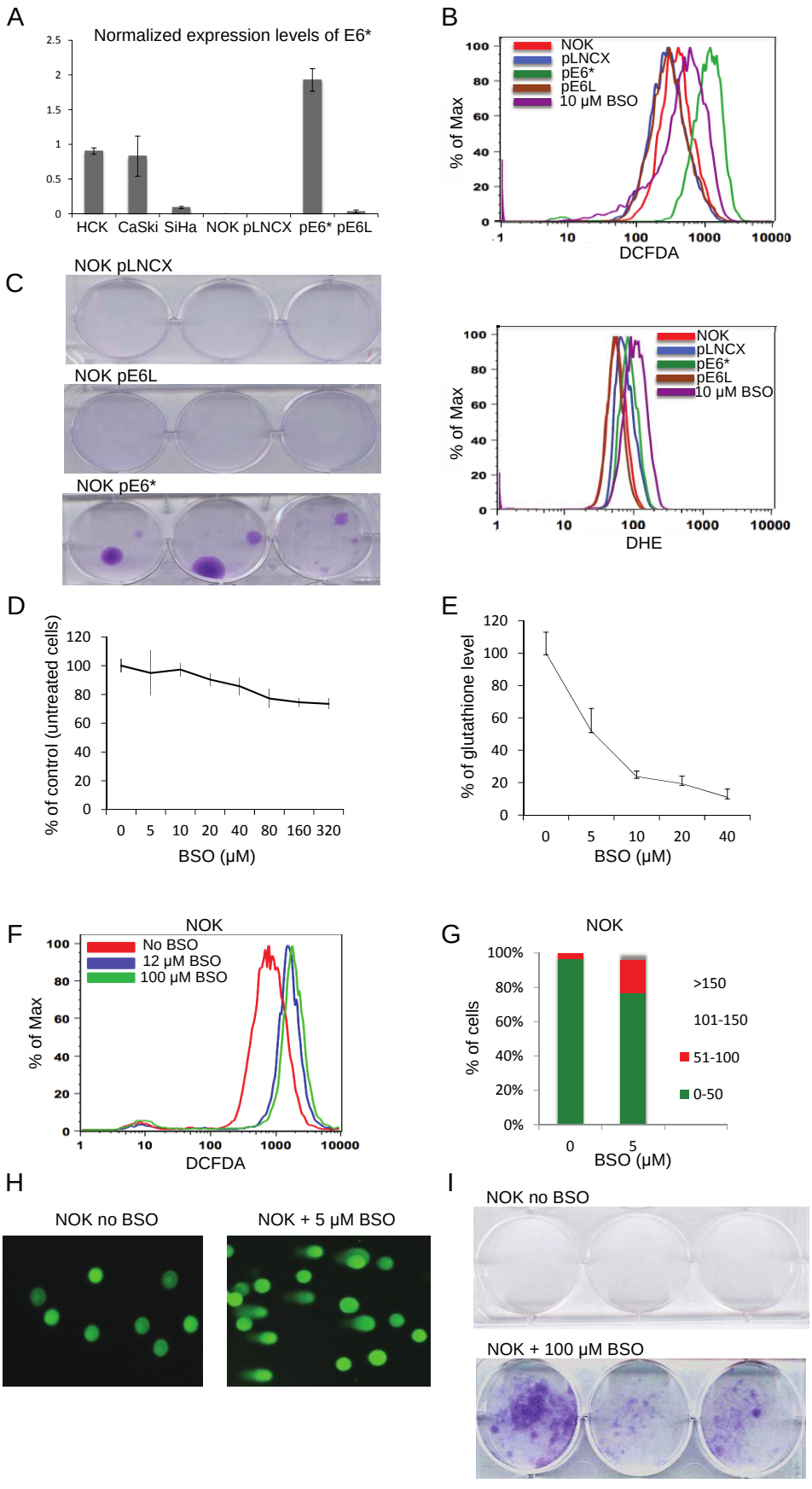
A
Normalized expression levels of E6*
HCK CaSki SiHa NOK pLNCX pE6* pE6L
B
NOK
pLNCX
pE6*
pE6L
10 μM BSO
% of Max
DCFDA
C
NOK pLNCX
NOK pE6L
NOK pE6*
NOK
pLNCX
pE6*
pE6L
10 μM BSO
% of Max
DHE
D
% of control (untreated cells)
BSO (μM)
E
% of glutathione level
BSO (μM)
F
NOK
No BSO
12 μM BSO
100 μM BSO
% of Max
DCFDA
G
NOK
% of cells
>150
101-150
51-100
0-50
BSO (μM)
H
NOK no BSO
NOK + 5 μM BSO
I
NOK no BSO
NOK + 100 μM BSO

Figure 4. OS, induced by either overexpression of E6* or by glutathione depletion, increases DNA damage and the frequency of plasmid DNA integration into NOK cells. **(A)** The expression levels of E6* transcripts in HCK, CaSki, SiHa, and NOK cell lines stably expressing either E6* alone (pE6*), E6 large (pE6L), or the empty vector (pLNCX) were analysed by qRT-PCR. E6* transcript expression levels were normalized by PGK gene expression. **(B)** ROS levels in NOK-derived stable cell lines and NOK treated with 10 μM BSO were analysed by flow cytometry using DCFDA (upper panel) and DHE (lower panel) as described in Material and Methods. **(C)** 10^6 cells per well of NOK-derived cell lines stably transduced with E6*, E6 large or the vector control were transfected with the pMetLuc *puro* plasmid. To normalize for transfection efficiency, media was collected 48 h post-transfection and the expression of secreted MetLuc was measured. Selection of cells resistant to *puromycin* (5 μg/ml) was performed for 3 weeks, and the colonies produced were stained with crystal violet. **(D)** NOK cells (10^4 cells per well) were plated onto a 96 - well plate, and cells were treated with BSO in triplicate at the indicated concentrations for 48 hours. Cell viability was estimated using the crystal violet assay. Cell viability without BSO treatment was set at 100%. **(E)** $5x10^4$ NOK cells were plated onto 24-well plates. After attachment, cells were treated with the indicated concentrations of BSO for 48 h. The resulting level of glutathione was measured using the Promega GSH-Glo™ glutathione assay kit according to the manufacturer's protocol. Measurements were performed in triplicate, and the glutathione level in untreated cells was set at 100%. **(F)** The level of ROS in NOK cells treated with the indicated concentrations of BSO for 48 h was estimated by flow cytometry following DCFDA staining. **(G)** and **(H)** NOK cells treated or untreated with 5 μM of BSO for 9 days, and the DNA damage was measured using the comet assay as described in Materials and Methods. **(G)** 100 cells were counted per cell line and the percentage of cells with each tail length was calculated. **(H)** Representative comet images of NOK cells untreated and treated with 5 μM BSO, visualized by microscopy following alkaline electrophoresis. **(I)** 6 $x10^6$ cells were transfected with the pMetLuc *puro* plasmid, then plated onto a 6 well plate. After 24 hrs, 3 wells were treated with 100 μM of BSO for 48 h. Selection of cells resistant to *puromycin* (5 μg/ml) was performed for 3 weeks and the resulting colonies were stained with crystal violet.

OS Induced by Glutathione Depletion Increases DNA Damage in HCK Cells Containing Episomal HPV16

The results described above indicate that chronic OS exogenously induced by BSO increases the rate at which plasmid DNA integrates into NOK cells. To determine if OS stress is also able to promote the integration of episomal HPV into the genome of the host, we employed HPV-positive human cervical keratinocytes, HCK, in which the HPV genome is normally maintained in an episomal state. These cells were kindly provided by Aloysius J. Klingelhutz (Sprague et al., 2002). First, we determined the concentration range of BSO that was capable of increasing ROS levels without affecting cell viability in HCK cells. We found that BSO concentrations within the 5 μM to 320 μM range did not affect viability (Figure 5A), however, such BSO treatments were accompanied by decreases in glutathione levels (Figure 5B). Furthermore, concentrations of 50 and 100 μM BSO did significantly increase ROS levels in HCK cells after 48 hours of treatment (Figure 5C).

DNA damage in HCK cells following treatment with 100 μM BSO treatment for 8 days was evaluated using the Comet assay. The results presented in Figure 5D show that the relative number of cells with longer tail lengths was increased after BSO treatment, as compared to the untreated group. A representative comet assay is shown in Figure 5E. These results demonstrate that exogenous application of the glutathione depleting agent BSO can induce OS and increase DNA damage in HCK cells containing episomal HPV16.

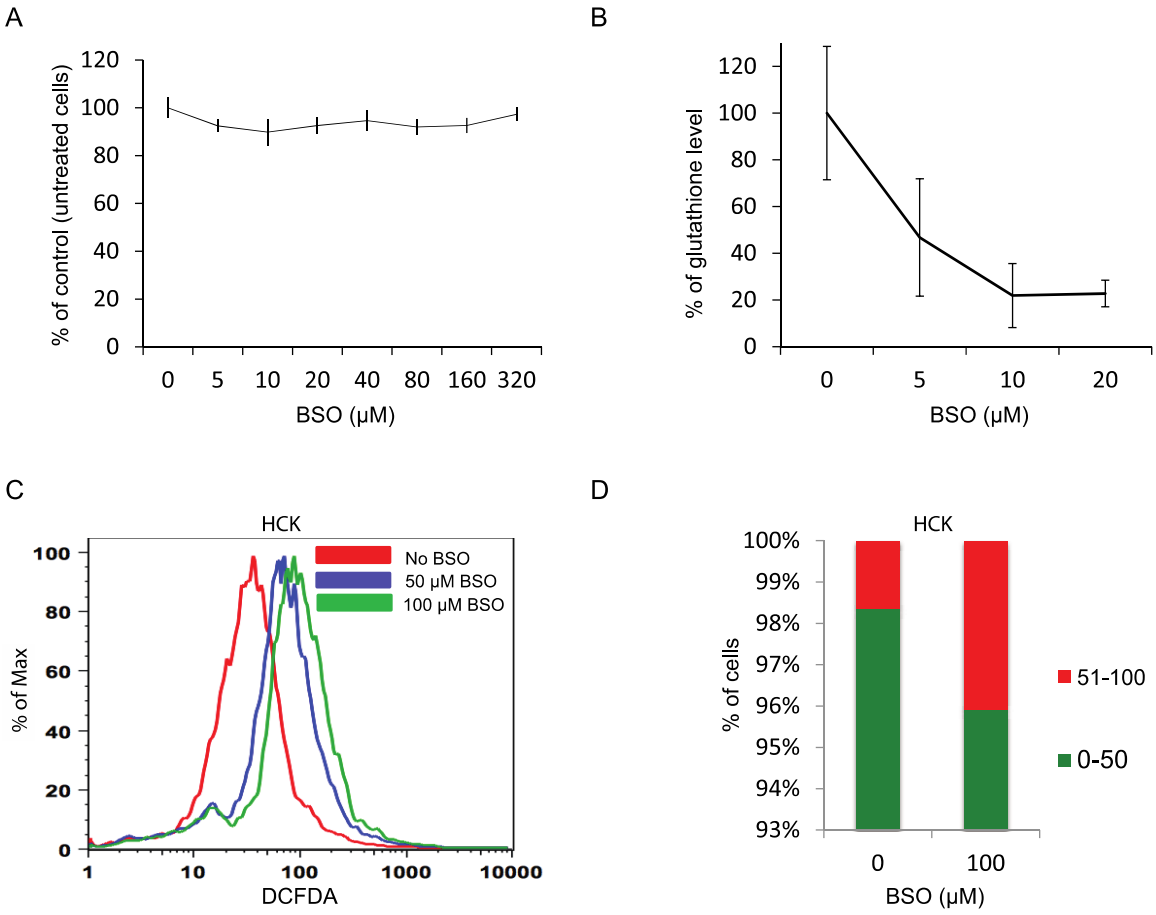
A
% of control (untreated cells)
120
100
80
60
40
20
0
0 5 10 20 40 80 160 320
BSO (μM)
B
% of glutathione level
120
100
80
60
40
20
0
0 5 10 20
BSO (μM)
C
HCK
No BSO
50 μM BSO
100 μM BSO
% of Max
100
80
60
40
20
0
1 10 100 1000 10000
DCFDA
D
HCK
% of cells
100%
99%
98%
97%
96%
95%
94%
93%
0 100
BSO (μM)
51-100
0-50
E
HCK no BSO
HCK + 100 μM BSO

Figure 5. OS induced by glutathione depletion increases DNA damage in HCK cells containing episomal HPV16. **(A)** 10^4 cells per well of HCK cells were plated onto a 96 - well plate. Cells were treated with BSO in triplicate at the indicated concentrations for 48 hours. Cell viability was estimated by the crystal violet assay, with cell viability without BSO treatment set at 100%. **(B)** 5 x 10^4 HCK cells were plated onto 24-well plates. After attachment, cells were treated with the indicated concentrations of BSO for 48 h. The level of glutathione was measured using the Promega GSH-Glo™ glutathione assay kit according to protocol. Measurements were performed in triplicate, and the glutathione level of untreated cells was set at 100%. **(C)** The level of ROS in HCK cells untreated or treated with the indicated concentrations of BSO for 48 h was estimated by flow cytometry following DCFDA staining. **(D)** and **(E)** HCK cells, were untreated or treated with 100 μM BSO for 8 days, then DNA damage was measured by the comet assay as described in Material and Methods. **(D)** 100 cells were counted per cell line and the percentage of cells with each tail length was calculated. **(E)** Representative comet images of HCK cells untreated or treated with 100 μM BSO, visualized by microscopy following alkaline electrophoresis.

Chronic OS Increases the Frequency of HPV16 Genome Integration in HCK Cells Containing Episomal HPV16

To determine whether the DNA damage induced by chronic OS results in an increase in the frequency with which the HPV episome integrates into the genome, we employed the Amplification of Papillomavirus Oncogene Transcripts (APOT) assay. This assay detects fusion forms in integrated viral oncogene transcripts (Figure 6A) (Klaes et al., 1999). HCK cells were treated with BSO, and samples of treated cells were collected at days 10 and 17. Samples of untreated cells were collected, and all samples were kept frozen at -80°C prior to RNA isolation and performance of the assay. Results from the APOT assay are shown in Figure 6B. RNA isolated from CaSki and SiHa cells were used as controls for the integrated HPV genome, since both cell lines are known to contain integrated HPV16 (Meissner, 1999).

An episomal HPV-derived transcript produces a 1050 bp product when assessed with a 5'-primer specific for the E7 region and a 3'-primer that binds to the poly-A tail region. If HPV is integrated, however, these primers will yield evidence of a fused transcript containing both HPV and human sequences. Hence, additional bands of different sizes will be observed (Klaes et al., 1999). The results of our APOT assay showed that PCR products from integration-derived transcripts in CaSki and SiHa cells were indeed of different lengths than the episomally-derived 1050 bp, showing that APOT detect fused transcripts (Meissner, 1999) (Figure 6B, right two lanes). In HCK cells untreated with BSO, however, the most abundant band was found to be 1050 bp, confirming our expectation and previous reports that this cell line carries the episomal form of the virus (Sprague et al., 2002) (Figure 6B, left-most lane). Treatment with BSO

for either 10 or 17 days led to the appearance of 3 additional bands of lesser size (Figure 6B). It is worth noting that the intensity of these additional bands was stronger at day 17 than at day 10 (Figure 6B, lane 3 *vs* lane 2). These results suggest that integration-derived HPV transcripts do appear after long-term BSO treatment, consistent with our working model stating that chronic OS increases DNA damage and hence episomal integration. To determine whether these transcripts were indeed derived from integrated HPV, these additional bands of less than 1050 bp were cloned and sequenced. These additional bands contained chimeric sequences composed of HPV viral sequences together with cellular sequences. Direct sequencing showed that in each case, the breakpoints in HPV occurred in the region of the E7 gene, while the 3 human insertions occurred within 3 separate genes, CUX2, JRKL-AS1, and tRNaseZL-interacting RNA B1. Together, these data indicate that treatment with BSO, which increased the level of OS and DNA damage in these episome-harboring cells, led to 3 independent integration events in which episomal HPV was inserted into the host genome, and support a chain of events leading from the induction of oxidative stress, to DNA damage, and finally, to viral integration.

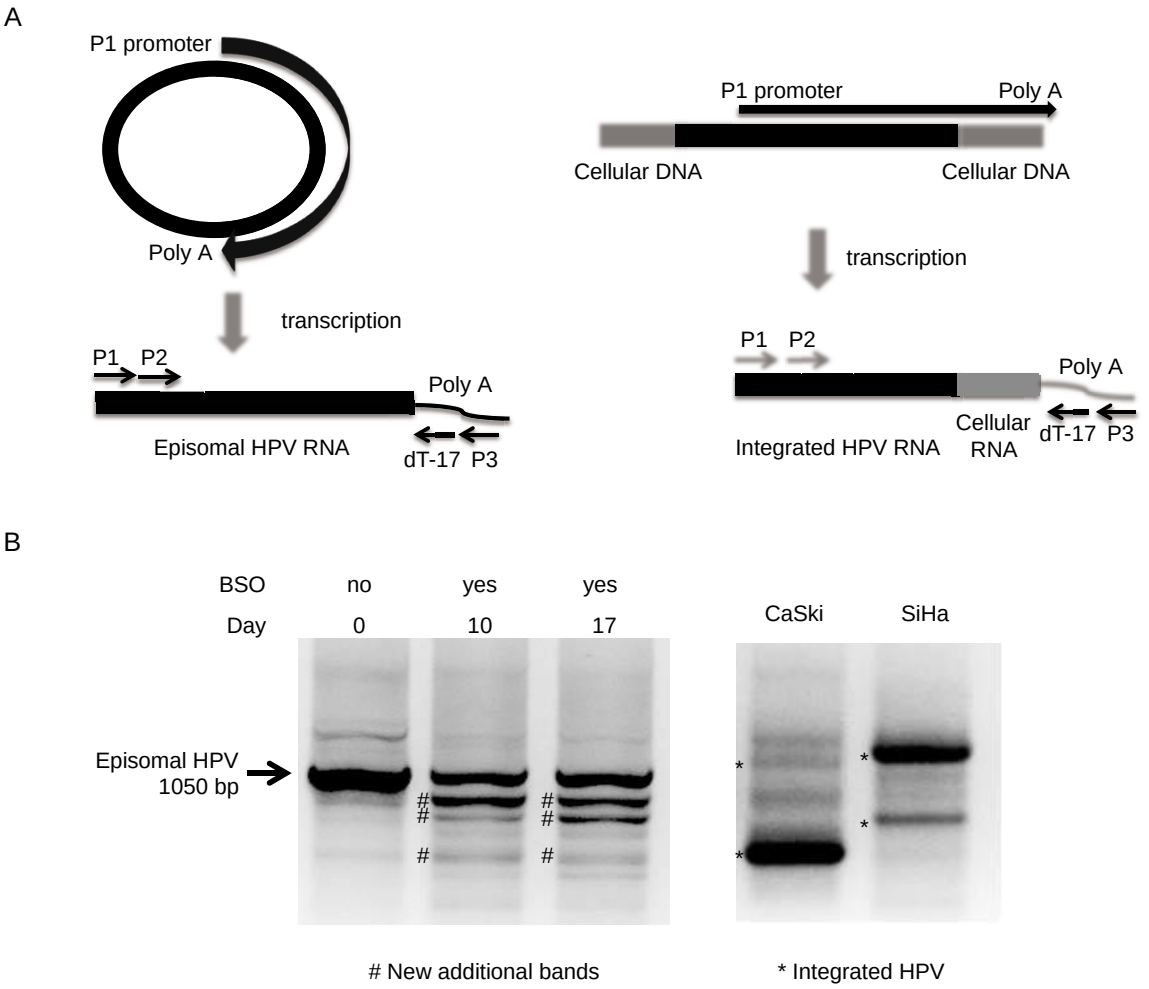

Figure 6. Chronic OS increases the frequency of HPV16 genome integration in HCK cells containing episomal HPV16. **(A)** Scheme showing the Amplification of Papillomavirus Oncogene Transcripts (APOT) assay. Transcripts derived from either episomal (left) or integrated (right) HPV16 forms, as well as the location of primers used for the Reverse Transcriptase Reaction ($(dT)_{17}$-p3) for PCR (p1, p2, p3) are indicated (modified from *Ruediger Klaes, Stefan M. et al.* (Klaes et al., 1999)). **(B)** HCK were treated with 5 µm BSO for 10 and 17 days. RNA from these cells, together with that from untreated cells (days 0) was isolated, then used to perform the APOT assay as described in Material and Methods. RNAs isolated from CaSki and SiHa cells were used as controls for the integrated HPV genome. * labels the integrated HPV genome. # labels new additional bands corresponding to transcripts from integrated forms of HPV16. cDNA was synthetized using oligo-dT, and nested PCR was performed using primers specific for E7 and poly-A.

Discussion

In the work reported here, we tested the hypothesis that virus-mediated and/or environmental factors can induce chronic OS that contributes to DNA integration. Consistent with our working model, we demonstrated that indeed, chronic OS increases the integration rate of both plasmid DNA and the HR HPV genome.

Integration of plasmid DNA occurs in response to OS induction, as assessed by a clonogenic assay (Figures 2A, 2B, Figures 3C, 3D, Figures 4C, 4I), as does HPV genome integration, as shown by APOT analysis (Figure 6B). These results are important because they demonstrate, for the first time, that the frequency of HPV integration can be manipulated by modulating levels of OS. It is generally accepted that high levels of ROS contribute to carcinogenesis by damaging DNA, RNA, proteins, and lipids, leading to cellular toxicity (reviewed in (Williams et al., 2011)). With respect to DNA, OS can induce DNA damage that results in apurinic/apyrimidinic (abasic) DNA sites, oxidized purines and pyrimidines, single-strand breaks (SSB) and double-strand breaks (DSB). These events further lead to chromosomal rearrangements and point mutations (Y. Chen et al., 2014; Kryston et al., 2011). With regards to DNA viruses, OS is particularly important in the context of HPV DNA integration, as ROS and reactive nitrogen species (RNS) have the potential to create the DSBs (Wei et al., 2009; Ziech et al., 2011) that can in turn enable viral integration. Our ability to induce HPV integration provides us with a novel tool to experimentally study the initial steps of carcinogenesis using models that closely resemble an actual HPV infection.

Among the different mechanisms of viral carcinogenesis, integration of the viral genome into the host genome plays a critical role during the process of transformation by

several oncogenic viruses including HBV, HPV, and Merkel cell polyomavirus (MCV). The connection between HBV integration and carcinogenesis is well accepted (Brechot et al., 1980; Shafritz et al., 1981), though the exact role of integration in Merkel cell carcinoma (MCC) carcinogenesis requires further study (reviewed in (Y. Chen et al., 2014)). The connection between HPV integration and carcinogenesis is also widely accepted (M. Pett & Coleman, 2007), and is largely based on analyses of patient tumor samples, as such cells present with integrated HPV at a very high frequency. However, this focus on fully transformed tissues limits the conclusions that can be drawn regarding factors that influence the integration events occurring prior to tumor development. Studies of tumor specimens indicate a lack of targeted disruption or functional alterations of critical cellular genes by the integrated viral sequences. Instead, most studies agree that integration occurs most frequently in common fragile sites (CFSs) (Wentzensen et al., 2004) and in intronic sequences of human cellular genes (Xu et al., 2013) within (or adjacent to) oncogenes (Wentzensen et al., 2004).

The three integration events we identified all involve a fusion between the E7 oncogene and a cellular sequence, with none of the three representing the type of integration event (between E2 and a cellular sequence) often observed in tumors. We therefore suggest that the integration of HPV genome into the host genome may be a much more frequent event than the initiation of transformation, and furthermore suggest that in most cases, this process is random and neutral in the context of cellular transformation. Consistent with our findings, a study from L. Turek's lab also shows that some identified HPV16 integration loci from head and neck cancer samples are involved in control of cell growth phenotype and oncogenes (16%), while other integration loci are

neutral and varied, suggesting that not every integration of the virus will lead to carcinogenesis. The functional role of these viral integration events in the course of oncogenic progression remains to be defined (Lace et al., 2011).

Integration occurs rarely and represents a dead-end for the virus life cycle. However, accumulating evidence indicates that the machinery for HPV integration is likely provided by the HPV replication process (McKinney, Hussmann, & McBride, 2015), by way of activating the DNA Damage Response (DDR). Several DNA viruses, including HPV, have been shown to activate and utilize DNA repair mechanisms to enhance their own replication (review (Lilley, Schwartz, & Weitzman, 2007)). This is a reasonable link, since there is overlap between enzymes that mediate repair and those that participate in DNA replication. HPV viral DNA replication occurs in the nuclear foci, and expression of the E1 and E2 replication proteins is sufficient for the formation of replication foci that recruit components of the DDR (Fradet-Turcotte et al., 2011; Reinson et al., 2013; Sakakibara, Mitra, & McBride, 2011; Swindle et al., 1999). HPVs enter the nucleus and initiate replication and transcription programs adjacent to nuclear domain 10 (ND10) bodies, which are associated with regions of DNA damage (Day, Baker, Lowy, & Schiller, 2004; Everett, 2006). Both E7 (Moody & Laimins, 2009) and E1 (Sakakibara et al., 2011) expression have been shown to activate the ATM-mediated DNA damage response. Although E6 inactivates the p53-mediated DNA damage repair pathway, p53-independent forms of DNA damage repair continue to function (Moody & Laimins, 2009) and thereby support viral replication. Following DNA damage, recruitment of the repair apparatus to the linear viral episome and/or breaks in the host genome likely occurs. The recruitment of DNA damage repair complexes ensures the

availability of ligases that can reconnect the recombined host and virus sequences, creating a microenvironment conducive to viral integration. In cases where the E1 and/or E2 regions are disrupted, these replication proteins are not expressed from integrated genomes but would be expressed from co-replicating, extrachromosomal HPV genomes, thereby inducing recruitment of DDR proteins to the integration loci, resulting in onion skin replication and promotion of genetic instability (Kadaja, Isok-Paas, Laos, Ustav, & Ustav, 2009; Kadaja et al., 2007). Any modification that results in increased expression of the E6 and E7 oncoproteins is anticipated to further stimulate genetic instability and promote carcinogenesis (McKinney et al., 2015). The DDR can be induced by exogenous factors (Giglia-Mari, Zotter, & Vermeulen, 2011) and, as noted above, is likely involved in HPV integration.

In this current report, we showed that DNA damage induced through exogenous chronic OS increased integration of the HPV genome (Figure 5A-E, 6B). DNA damage, and the resulting DDR, can also be induced by virus-derived factors, as the ability to damage DNA has been reported for E2, E6, E6* and E7 (Bermudez-Morales et al., 2009; Duensing & Munger, 2002; Williams et al., 2014b). In fact, our results demonstrated that E6* expression can induce DNA damage through increasing ROS levels and thereby promote foreign DNA integration (Figures 2A, 2B, Figures 3C, 3D, Figures 4C, 4I). Factors other than OS can also promote integration of the viral genome. E6 and E7 expression have been shown to induce genome instability and contribute to an increased integration rate (Kessis et al., 1996); in addition, viral load and persistence have been demonstrated to promote integration (Moberg, Gustavsson, Wilander, & Gyllensten, 2005; M. Pett & Coleman, 2007).

Importantly, we were able to successfully decrease the integration frequency by using antioxidants (resveratrol and vitamin E). These results (Figures 3E-H) are significant because they suggest that if chronic OS is indeed a factor promoting HR HPV integration, we have the potential to develop prophylactic and therapeutic strategies designed to prevent this initial step in carcinogenesis. Reduction of risk factors associated with chronic OS should be therefore considered as a prophylactic approach for the prevention of cervical cancer. Theoretically, therapeutic or dietary measures aimed at reducing oxidative stress could decrease oxidative stress in already-infected cells (Garcia-Closas et al., 2005), and thereby diminish the risk of viral integration. In this report, we used the antioxidants vitamin E and resveratrol to reduce levels of cellular oxidative stress, which in turn reduced the integration of foreign DNA into U2OS cells. Our findings suggest that dietary antioxidants may be able to supplement the activity of endogenous antioxidants found in normal cells and fortify them against challenges posed by increased levels of ROS. In fact, studies have shown that several antioxidants are reduced in the circulation of cervical cancer patients (Manju et al., 2002). A deficiency in antioxidant vitamins and/or other dietary components may, therefore, contribute to the increased prevalence of cervical cancer observed in women with a low socioeconomic status (Manju et al., 2002). Possible benefits from dietary antioxidant consumption remain under discussion (Garcia-Closas et al., 2005), and additional work is needed to test the effect of antioxidant therapies as a new approach for decreasing the probability of integration and thereby reducing cervical cancer incidence.

Overall, our results demonstrate that chronic OS can induce DNA damage and increase the frequency of HPV integration into the host genome, and in this way

contribute to cervical carcinogenesis. Future strategies may focus on assessing cancer risk by screening for OS levels, then applying therapies directed toward OS, thereby decreasing the integration rate, and ultimately, carcinogenesis.

CHAPTER THREE

IDENTIFICATION OF INTEGRATION SITES BY NEXT GENERATION SEQUENCING (NGS) BASED ANALYSIS

Introduction

Previously, we had employed the Amplification of Papillomavirus Oncogene Transcripts (APOT) method to identify and characterize integration events in HCK cells containing episomal HPV16 that occur after exposure to chronic OS. The APOT data showed that chronic OS increases the integration rate of the HPV genome into the genome of these HCK cells . However, the APOT method suffers from certain limitations: for example not all insertions will be detected, as the events detected depend upon the primers chosen. Therefore, we developed a new method, based on Next Generation Sequencing (NGS) technology, to quantify and characterize the frequency and sites of HPV integration. The precise determination of integration sites using this NGS technique not only permits us to test our hypothesis that chronic OS promotes HPV integration, but also will shed light on the relationship between virus integration and the initial steps of transformation. Careful quantitative analysis of individual integration sites, immortalization and transformation events, coupled with detailed characterization of the sites of integration in both viral and human DNAs, will provide information on the forces and factors that influence viral integration.

Materials and Methods

Reagents

L-Buthionine-sulfoximine (BSO) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solution, at a concentration of 100 mM, was prepared in phosphate-buffered saline (PBS) and kept at -20°C.

Cell Culture

Human cervical keratinocytes (HCK) containing episomal HPV16, kindly provided by Dr. Aloysius J. Klingelhutz (Sprague et al., 2002), were grown in E-media (Wu et al., 1982) with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich, St. Louis, MO).

Preparation of HPV-specific SureSelect Libraries

To evaluate the integration state of HPV16 molecules and to detect the exact sites of integration for both human and virus DNAs, we employed the target enrichment approach using SureSelect DNA target baits synthesized by Agilent Technologies (Santa Clara, CA). Briefly, a pool of up to 16 indexed libraries prepared from the 16 DNA samples were sequenced simultaneously. DNA isolated from cell samples was hybridized to RNA SureSelect RNA baits that cover the entire HPV 8Kb genome to capture HPV-derived sequences from the total cellular DNA, which were subjected to sequencing at the LLU Center for Genomics. We used CaSki DNA as a reference to evaluate the method since the HPV status and integration events in this cell line have been fully analyzed previously (Akagi et al., 2014).

NGS Data Analysis

Three types of sequences were detected and analyzed: those including only HPV sequences, those including only human sequences, and those containing fused HPV plus human DNA sequences which include insertion sites. The NGS data were analyzed by VirusFinder freeware (Q. Wang, Jia, & Zhao, 2013). In brief, reads were first aligned against the human genome sequence by Bowtie2 mapping to extract non-human reads, which then were mapped against HPV16 DNA using the same Bowtie2 program. The reads that contained HPV16 sequences were analyzed for the presence of potential breakpoints using SVDetect and CREST detection tools. Breakpoint sequences were considered indicators of HPV16 integration sites.

Results

Exogenous Factors Increase the Integration Rate of HPV 16 in HCK Cells Containing Episomal HPV16

We employed our newly-developed NGS-based approach to identify and characterize integration sites in HCK cells containing episomal HPV16 following exposure to exogenously induced chronic OS. HCK cells were subjected to treatment with or without BSO, and samples were collected at day 21 as shown in Figure 7. CaSki cells were used as a positive control for the integrated HPV genome, since they are known to contain multiple copies of integrated HPV16 (Meissner, 1999). We prepared the SureSelect DNA libraries as described in Materials and Methods. The cellular DNA fragments were hybridized to RNA SureSelect RNA baits that cover the entire HPV 8Kb genome, then isolated and subjected to simultaneous sequencing. The NGS data were analyzed by VirusFinder freeware, and the results are shown in Table 2. The total number

of integration events identified in CaSki cells was 44, which is similar to the number of integration events identified previously (Akagi et al., 2014). The total number of integration events we detected in HCK cells treated with BSO was 19, a significant increase as compared to those found in the control, untreated cells in which only 2 integration sites were detected. The results obtained by this NGS-based analysis is consistent with the results we found previously using the APOT method; specifically, both methods demonstrated that chronic OS induced by long-term BSO treatment increased the integration rate of the HPV16 genome.

Table 2. Deep sequence mapping and HPV integration across cell lines

Sample ID	Trimmed Reads (M)	Mapped to Human (M)	Mapped to HPV (M)	Unmapped Reads (M)	Total Split Reads	Intronic Sites	Intergenic Sites	Total
CaSki	27.19	0.13	26.36	0.7	2,918	16	28	44
HCK	16.28	8.75	6.14	1.39	1,575	1	1	2
HCK+BSO	17.87	10.78	6.51	0.57	6,629	10	9	19

Discussion

Our NGS-based analysis demonstrated that chronic OS, as induced by exogenous factors, can increase the integration rate of HPV 16 into HCK cells containing episomal HPV16, as outlined in Figure 7. This data is consistent with the results we observed previously using the APOT method.

However, the library preparation we employed includes PCR-based steps that can introduce false positive HPV-human amplification PCR products. For this reason, we plan to verify the candidate insertion sites through independent PCR analysis (Figure 8) in future experiments.

HPV integration sites are distributed randomly throughout the host genome, without a single region predominating (Wentzensen et al., 2004). However, 38% of 192 integrants were found in known common fragile sites (CFSs), and there was no evidence to suggest targeted disruption or functional alteration of critical cellular genes by the integrated viral sequences (Wentzensen et al., 2004). However, some studies have demonstrated that high-risk HPV integration has occurred within or adjacent to known oncogenes, most commonly within intronic sequences (Ferber et al., 2003; Thorland et al., 2003; Wentzensen et al., 2002; Wentzensen et al., 2004). For example, the region of the MYC gene at chromosomal band 8q24 is a frequently observed integration site in HPV18-positive cervical cancer (Ferber et al., 2003; Peter et al., 2006; Wentzensen et al., 2002). NGS-based methods now provide a very efficient method to map HPV integration sites. One NGS study, for example, showed that the 3'-breakpoints of integrated HPV16 DNA distribute preferentially within the early region E1-PAE segment in HPV 16. This indicates the importance of deregulated viral oncogene expression for carcinogenesis (Xu

et al., 2013). Interestingly, about half of the mapped HPV16 integration sites directly target human cellular genes (Xu et al., 2013). A genome-wide analysis of HPV integration in human cancer cell lines mapped HPV integrants in human cancer genomes and showed that they directly flank genomic structural variations, including focal amplifications, rearrangements, deletions, and/or translocations. These genomic alterations frequently disrupted the expression and structure of neighboring genes involved in oncogenesis, and resulted in amplification and expression of E6 and E7 (Akagi et al., 2014). For example, the HPV integrant cluster causing homozygous deletion of DIAPH2 may promote chromosomal instability *via* misalignment of sister chromatids during metaphase (Cheng et al., 2011). Moreover, HPV integrants introduced aberrant promoters that resulted in expression of a highly expressed, truncated form of p63 protein containing a domain known to inhibit the pro-apoptotic protein, TAp63 (Serber et al., 2002). The disruption of such genes by HPV integrants is likely of biological significance, given the established functions of affected genes.

These studies suggest that the insertional mutagenesis of the host genome may play a role in cervical carcinogenesis. However, many of the previous publications using NGS focus on identifying genotype and determining HPV load, rather than on identifying sites of integration (Conway et al., 2012; Meiring et al., 2012). Our results here showed that we can identify HPV integration events and sites by using NGS based analysis. Further studies are needed to verify the candidate integration sites and assess any resulting genomic mutations.

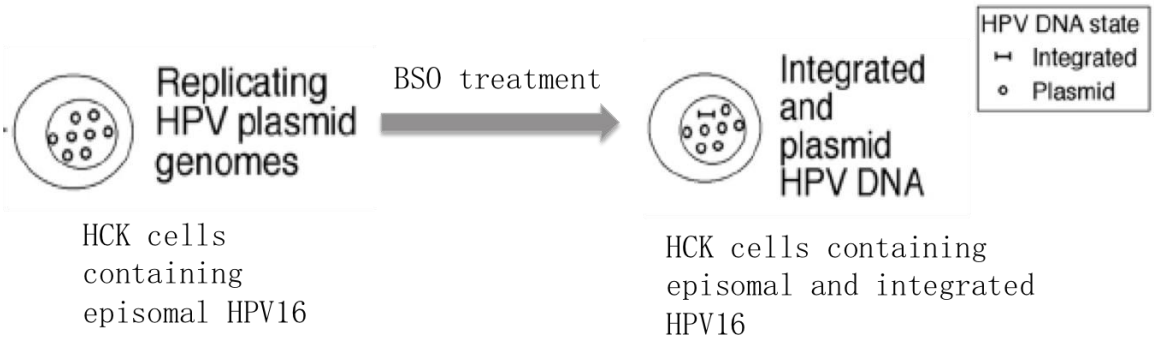

Figure 7. Chronic OS induced by long term BSO treatment increases the integration rate of HPV 16 in HCK cells containing episomal HPV16.

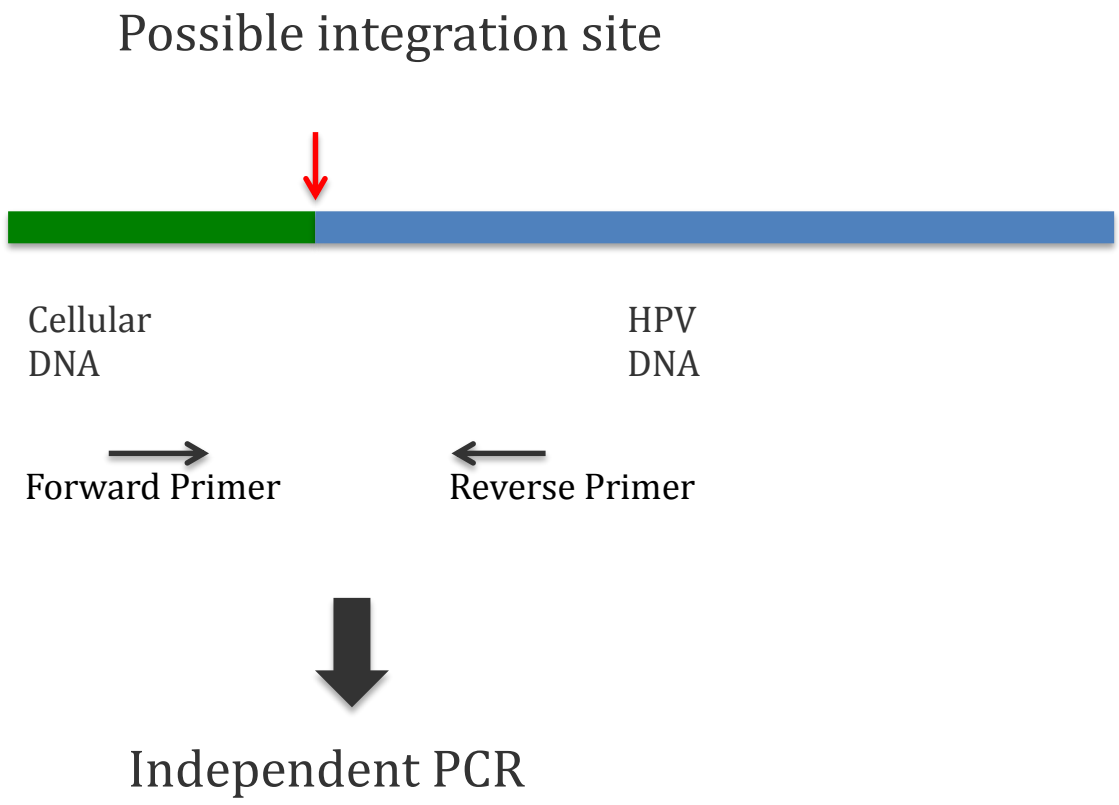

Figure 8. Verification of the candidate insertion sites by independent PCR analysis.

CHAPTER FOUR

OXIDATIVE STRESS MAY INFLUENCE SUSCEPTIBILITY TO CERVICAL CANCER: CERVICAL SPECIMENS DISPLAY VARIABILITY IN LEVELS OF ROS AND DNA DAMAGE

Running title: ROS levels vary between cervical specimens

Maria Filippova, Yan Chen Wongworawat, Sam Siddighi, Sveta Bashkirova, Penelope J. Duerksen-Hughes

Oxidative stress may influence susceptibility to cervical cancer: Cervical specimens display variability in levels of ROS and DNA damage. Oxi Med Cell Longev. 2016; Under Review.

Abstract

High risk human papillomaviruses (HPV) are the causative agents of cervical cancer. However, not all infected women develop cervical cancer. Cervical tumorigenesis is characterized by a multifactorial etiology, with oxidative stress (OS) likely playing a major role. In addition to exogenous sources, metabolic processes also contribute to OS. In principle, variability in levels of cervical OS has the potential to influence the likelihood of conversion to cervical cancer. To ask whether such variability indeed existed, we assessed ROS levels in normal, non-cancer cervical tissue, and demonstrated a greater than three-fold variability between tissues isolated from different women. Primary keratinocytes were also isolated and cultured, and displayed very similar levels of ROS as those observed in the corresponding tissue. Importantly, we demonstrated that the level of DNA damage in cultured cells also mirrored the level of ROS. Finally, we found that differences in the levels of ROS may be in part regulated by differences in the expression of antioxidant enzymes. Understanding the factors and mechanisms through which some, though not most, individuals develop cervical cancer has the potential to enable the development of approaches that make the conversion of HPV infection to cancer development even more rare.

Introduction

Cervical cancer is the second most common cancer in women, and the fourth most common cause of cancer-related death in women worldwide (Bernard W. Stewart, 2014; Parkin & Bray, 2006). High risk human papilloma virus (HPV) infection is well-established as the causative agent of cervical cancer (Ahn et al., 2003), and at least 85% of premalignant and 90% of malignant squamous lesions in the uterine cervix test HPV DNA positive (Smith et al., 2007). Infections with high-risk types of human papillomaviruses (HR HPV) are extremely common; approximately 20 million Americans are currently infected, about 15% of the total population, with another 6 million new infections occurring each year. In more than 90% of these cases, the infection is cleared by the immune system within two years, especially in younger women and adolescents (Franco et al., 1999; Molano et al., 2003). However, a relatively small subset of infections persists, and of these, some progress to malignancy. In particular, approximately 0.3% - 0.5% of pap smear specimens are typically diagnosed with carcinoma *in situ* (Banik, Bhattacharjee, Ahamad, & Rahman, 2011), indicating that not all infected women develop cervical cancer, and that, in fact, the vast majority will not. Understanding the factors and mechanisms through which some, though not most, individuals develop cervical cancer has the potential to enable the development of approaches that make the conversion of HPV infection to cancer development even more rare.

Because HPV infection alone is not sufficient to induce cervical cancer, cervical tumorigenesis is clearly characterized by a multifactorial etiology (H. W. Haverkos, 2005), with oxidative stress (OS) likely playing a major role in the process (De Marco,

2013; De Marco et al., 2012). A number of clinical conditions have implicated OS as a contributory factor, including chronic inflammation, diabetes, atherosclerosis, ischemia-reperfusion injury and of particular interest, malignancies of different origins (Cooke, Evans, Dizdaroglu, & Lunec, 2003; Halliwell, 2007; Waris & Ahsan, 2006). In the case of cervical cancer, known risk factors in addition to HPV infection include smoking (H. W. Haverkos, Soon, Steckley, & Pickworth, 2003), tar-exposure (H. Haverkos, Rohrer, & Pickworth, 2000), co-infection with other viruses such as herpes simplex virus-2 (H. Haverkos et al., 2000), co-infection with other STD, lifestyle, and diet (Munoz, Castellsague, de Gonzalez, & Gissmann, 2006). Each of these factors can induce OS by one mechanism or another. For example, smoking was shown to induce OS by increasing the level of free radicals (Plummer, Herrero, Franceschi, Meijer, Snijders, Bosch, de Sanjose, Munoz, et al., 2003; Tollefson et al., 2010). Infections and co-infections that induce inflammation also result in increases in the levels of reactive oxygen species (ROS), as the innate immune defense system utilizes the induction of OS as a powerful weapon against pathogens (reviewed in (Y. Chen et al., 2014; Williams et al., 2011)). Elevated levels of reactive oxygen species (ROS) induce damage to DNA, lipids and proteins, damage to tumor suppressor genes, and enhanced expression of proto-oncogenes (Halliwell, 2007; Klaunig, Kamendulis, & Hocevar, 2010). With regards to DNA, the ability of reactive nitrate and oxygen species to damage DNA, thereby leading to single and double-strand breaks, larger-scale damage and cancer, is well known (Cooke et al., 2003). For example, lungs of cigarette smokers contain two to three fold higher levels of the modified deoxynucleotide, 8-oxoguanine (Olinski et al., 1992), which

was shown to be induced by oxygen free radicals, resulting in inflammatory responses, fibrosis and tumor development (Zienolddiny, Ryberg, & Haugen, 2000).

In addition to exogenous sources of OS such as smoking and infection, metabolic processes can also contribute to the level of OS in living cells. Under normal circumstances, homeostasis of ROS is maintained by several mechanisms, including the genetic and epigenetic regulation of genes coding for proteins that function in pro-and anti-oxidant systems (D'Autreaux & Toledano, 2007; Ray, Huang, & Tsuji, 2012). Individual variability in the expression and/or function of such proteins and their regulators has the potential to translate into variability in levels of ROS, and in fact, the human population has been shown to be heterogeneous with regards to ROS levels (Agarwal, Saleh, & Bedaiwy, 2003; Dato et al., 2013; Salganik, 2001). While the role of exogenous factors in influencing the risk of cervical cancer is well documented (H. Haverkos et al., 2000; Munoz et al., 2006), available information on the influence of genetic/epigenetic factors on cervical cancer risk is fragmented. Most published studies have focused on finding correlations between particular genetic markers or mutations with cancer incidence by studying tissue that is already cancerous (Zhang, Zhang, Tian, Yang, & Wang, 2014). Interestingly, even the question of whether cervical cancer is or is not hereditary is still in debate. Some consider that cervical cancer is not hereditary because the causative agent for cancer is HPV, which is the same for virtually all cases (Lynch, Casey, Shaw, & Lynch, 1998). Another group believes that because genetic susceptibility to HPV exposure and/or infection appears to be important in determining the individual risk for developing HPV-mediated cancer, this cancer could be considered to have a hereditary component (Magnusson & Gyllensten, 2000).

We suggest that a high level of ROS in cervical tissue, which may result from a combination of external influences as well as internal, genetic/epigenetic factors, has the potential to contribute to the likelihood that a particular woman will develop cervical cancer. To assess this possibility, we characterized normal, non-cancer cervical tissue for levels of ROS, and demonstrated a greater than three-fold variability in the levels of ROS between tissues isolated from different women. Primary keratinocytes were also isolated and cultured from these specimens, and displayed levels of ROS that were very similar to those observed in the corresponding tissue. Importantly, we demonstrated that the level of DNA damage also mirrored the level of ROS in cultured cells. Finally, we found that differences in the level of ROS may be in part regulated by differences in the expression of antioxidant enzymes.

Material and Methods

Preparation of Cervical Specimens

Normal cervical specimens were collected from patients with uterovaginal pelvic organ prolapse following vaginal hysterectomy at the Loma Linda University Surgical Hospital. Samples were anonymized to prevent linkage to identifiable individual data. Waste tissues not needed for clinical analysis were collected and placed into 0.9% normal saline solution and transported to the laboratory. Each specimen was then dissected in cold PBS under sterile cell culture conditions. The transformation zone (TZ) and ectocervical (EC) regions were separated and divided into several parts for further use.

ROS Analysis - Cervical Tissue

Specimens isolated from the TZ and EC were weighed and transferred to glass vials containing PBS for homogenization. The volume of PBS used for each sample was calculated to yield 200 mg of tissue in 1 ml of PBS. Homogenates were centrifuged at 10,000 rpm for 5 min at 4^{o}C, after which the supernatant was transferred to a new eppendorf tube, then snap-frozen in liquid nitrogen and stored at $^{-}$80^{o}C until use. During homogenization, the free radicals will be released to the PBS according to manufacture instruction.

ROS levels in these homogenate supernatants were measured using the OxiSelect *In Vitro* ROS/RNS assay kit (Cell BioLabs, San Diego, CA) according to the manufacturer's protocol. Hydrogen peroxide (H_2O_2), hydroxyl (OH^-) and peroxyl (ROO^-) radicals were detected using dichlorodihydrofluorescin (DCFDA). Protein concentrations were measured with the Coomassie Plus Assay Reagent (Thermo Fisher Scientific, Rockford, IL) and used for normalization.

Isolation and Culturing of Normal Primary Cervical Keratinocytes

Primary keratinocytes from TZ and EC regions were isolated using the protocol "Isolation, Primary Culture, and Cryopreservation of Human Keratinocytes" from Thermo Fisher Scientific (Life Technologies). Cells were maintained in keratinocyte growth media, E-media (Piboonniyom et al., 2003), with the feeder layer provided by mouse NIH 3T3 fibroblasts treated with 4 µg/ml of Mitomycin C (Sigma-Aldrich, St. Louis, MO) for 4 h. To prevent keratinocyte differentiation, 10 µM of the ROCK pathway inhibitor, Y-27632 (Tocris, Bristol, UK), was included in the culture medium.

ROS Analysis – Cultured Keratinocytes

Intracellular generation of H_2O_2, OH^- and ROO^- radicals, as well as of superoxide (O_2^-), was estimated using either the 5-(and-6)-Carboxy-2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) or Dihydroethidium (DHE) membrane permeable probes (Thermo Fisher Scientific, Life Biosciences), respectively. Reagents were diluted into culture media, and then added to cells to a final concentration of 10 µM. After treatment, the cells were collected in 1X PBS and analyzed using the Becton-Dickinson FACSCalibur flow cytometer (Becton-Dickinson, San Francisco, CA). DCFDA was detected in the FL-1 channel, while DHE was detected in the FL-2 channel. Data was collected in log scale and analyzed using Flow-Jo software.

Immunoblot Assays

10^6 cells were lysed in 100 µl of Laemmli lysis buffer, and lysates were sonicated and separated by SDS-PAGE. After the transfer of protein onto PVDF membranes (Thermo Fisher Scientific, Life Technologies) and blocking of the membrane in Odyssey

Blocking Buffer (Li-COR, Lincoln, NE), primary antibodies were applied overnight. Monoclonal α-β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO), monoclonal α-superoxide dismutase1 (SOD1), α-glutathione reductase (GR) and α-glutathione peroxidase (Gpx ½) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal α-superoxide dismutase 2 (SOD2) antibodies from BD Biosciences (San Jose, CA). Secondary goat anti-mouse IRDye 800 CW (Li-COR, Lincoln, NE) was applied onto the membrane for 1 h, and the detection of signal was performed using Odyssey Imaging System (Li-COR, Lincoln, NE).

DNA Damage - 8-Oxyguanine Analysis

Avidin-FITC (Thermo Fisher Scentific, Life Technologies) was used to measure 8-oxoguanine levels in cells (Achanta & Huang, 2004). Cells were fixed in 1% paraformaldehyde and permeabilized in methanol. 10 µM of Avidin-FITC was applied to cells for 1 h, and after two washes, binding of the Avidin-FITC-conjugate to 8-oxoguanine in cells was monitored using flow cytometery (excitation 495 nm, emission 515 nm) on a BD FACS Calibur (BD, Franklin Lakes, NJ), then analyzed using Flow-Jo software. A total of 10,000 events were measured per sample.

Statistics

All measurements for ROS levels were performed in triplicate, and error bars on graphs represent standard deviations. The F-test was used to determine whether the variances between two variables was significant.

Results

Collection and Preparation of Cervical TZ and EC Specimens

Cervical tissue includes the endocervix, the transformation zone, and the ectocervix. The endocervical mucosa is lined with a single layer of columnar mucous cells within the endocervical canal, while the ectocervix is covered with nonkeratinized stratified squamous epithelium directed towards the vaginal portion of the cervix (Gray, Williams, & Bannister, 1995). The junction between these two types of epithelia is called the squamocolumnar junction (SCJ) (Beckmann & American College of Obstetricians and Gynecologists., 2014). In prepubertal females, the functional SCJ is present within the endocervical canal. Upon entering puberty, hormonal influences cause the columnar epithelium to extend outwards over the ectocervix as the cervix everts, and also cause the SCJ to move outwards onto the vaginal portion of the cervix (Beckmann & American College of Obstetricians and Gynecologists., 2014). The zone of unstable epithelium between original SCJ and the new SCJ, which is internal to the original SCJ, is called the transformation zone (TZ) (Beckmann & American College of Obstetricians and Gynecologists., 2014). The TZ is a highly active metaplastic tissue in which the single layer columnar epithelium is transformed into the multilayered squamous epithelium of the ectocervix (EC). The cells of this zone, which are organized as single layer epithelium, are potential targets for HPV infection (Schiffman, Castle, Jeronimo, Rodriguez, & Wacholder, 2007). If these TZ cells are infected with HPVs, they can become the primary sites for cervical intraepithelial neoplasia development after replacement by non-keratinized stratified squamous epithelium (Schiffman et al., 2007). Because these TZ cells are the likely targets for HPV, they were chosen for further

investigation. The corresponding EC cells were also analyzed, as they also play a role in the virus life cycle.

Normal cervical tissue, isolated from patients undergoing vaginal hysterectomy, was dissected in cold PBS. The TZ and EC portions were separated, and specimens were prepared for measuring tissue ROS levels and for isolating primary keratinocytes for subsequent *in vitro* culturing. Specimens from 19 patients were collected and subjected to analysis of ROS levels, while the comparison of ROS levels between TZ and EC tissues was performed on specimens isolated from 5 patients. Specimens isolated from 5 patients were employed for isolation and culturing of primary keratinocytes from the TZ and EC regions. The histology of the TZ and EC regions after dissection of specimen #20 are shown in Figure 9. The TZ region is characterized by single layer epithelium (Figure 9A), while the EC displays a morphology of multilayered squamous epithelium with basal/parabasal, intermediate and superficial layers (Figure 9B).

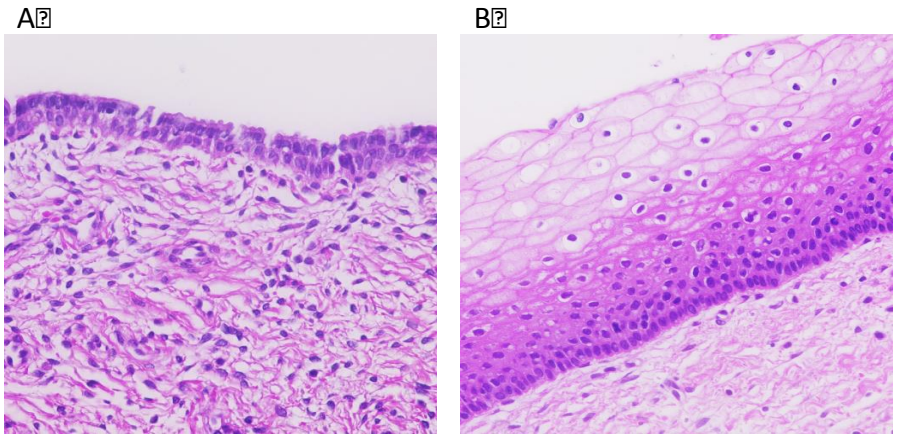

Figure 9. Representative specimen #20 showing TZ **(A)** and EC **(B)** tissues isolated from the cervix. After dissection, the tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin-eosin.

ROS Levels in Cervical TZ tissues Differ Within a Three-fold Range Between Patients

The observation that OS is a promoting factor for numerous pathologies, including several types of cancer, suggested that an excess of free radicals could also contribute to HPV-induced cervical tumorigenesis. In particular, it seemed possible that differential background levels of ROS in cervical cells, caused by some combination of differences in genetic/epigenetic backgrounds together with variable exposure to exogenous factors, might contribute to the likelihood that a particular HPV-infected woman might or might not progress to cancer. To assess how the level of ROS in cervical tissues from different women might vary, TZ specimens isolated from nineteen patients after vaginal hysterectomy were subjected to ROS analysis, and the level of H_2O_2, OH^- and ROO^- radicals was detected using DCFDA. The data obtained (Figure 10A) demonstrate significant variability between patients in the level of ROS found in their TZ. For example, the lowest level of ROS was noted in sample #15 (717 FLU/µg of protein), while the highest was noted in sample #16 (2334 FLU/µg protein). This represents a difference of more than 3-fold. ROS levels for the other samples are distributed between these values (Figure 10A).

As noted above, TZ cells are the primary targets for HPV infection. However, after replacement with non-keratinized stratified squamous epithelium, the HPV life cycle takes place in the multilayered squamous epithelium of the EC. We measured and compared ROS levels between single- and multilayered epithelium from the cervix, tissues that differ both functionally and structurally. Figure 10B shows the ROS levels observed in homogenates prepared from the TZ and EC regions of 5 specimens. Within each pair (TZ and EC), the ROS level in the TZ was higher than or approximately equal

to that seen in the EC. For example, in sample #16, the difference between the TZ and EC was more than two-fold, while in samples #17 and #20, there was no significant difference between the two. The ROS levels observed in the TZ and EC regions are likely to reflect contributions from genetic/epigenetic regulation of ROS levels, together with influences from exogenously-derived factors such as exposure to drugs, environmental factors and life style.

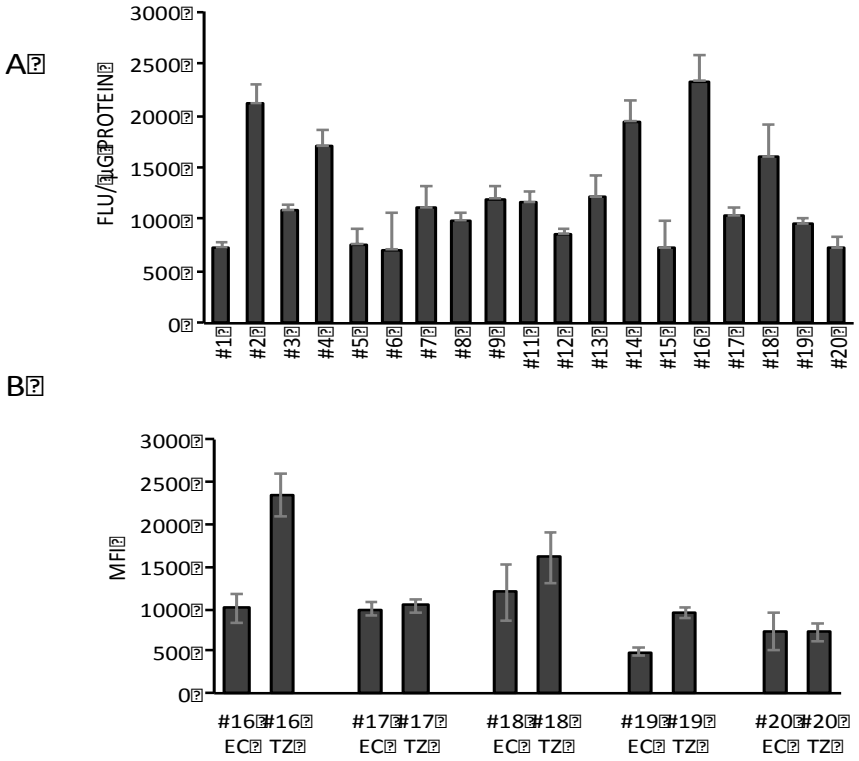

Figure 10. ROS levels in cervical TZ tissues. **(A)** ROS levels in cervical TZ tissues differ within a range of three-fold between patients. Homogenates were prepared from TZ tissues immediately after surgery, and ROS levels were assessed using the OxiSelect *In Vitro* ROS/RNS assay kit (Cell BioLabs) according to the manufacturer's protocol. Protein concentrations were measured with the Coomassie Plus Assay Reagent (Thermo Scientific) and used for normalization. ROS levels are represented as fluorescent light units (FLU)/µg protein. (**B)** Tissue homogenates prepared from the TZ have greater or equal levels of ROS than do homogenates prepared from the EC. The level of ROS in 5 TZ and EC tissue homogenates was measured as described in A). All measurements were performed in triplicate, and error bars represent the standard deviation.

Culture of Primary Keratinocytes

Dissection of the primary tissue enables us to obtain approximately 200 mg of TZ or EC tissues, which consists of a keratinocyte layer, along with fat and connective tissues. This amount of material is sufficient for only a limited number of studies. Therefore, in order to increase the amount of material available for investigations focused on areas such as the modeling of HPV infection, HPV integration and cellular transformation, we applied known procedures to isolate and culture primary keratinocytes from the EC and TZ cervical tissues. One property that frequently restricts keratinocyte production is the well-known fact that primary keratinocytes in culture undergo terminal differentiation after several divisions. We were able to overcome this restriction and create a culture that can divide indefinitely by blocking the ROCK pathway using the Y-27632 inhibitor of serine/threonine kinases ROCK-I and ROCK-II (McMullan et al., 2003). Application of ROCK inhibitors to keratinocytes has been shown to prevent terminal differentiation and induce cell division (McMullan et al., 2003). Using Y-27632, we were able to maintain the keratinocyte culture for 5 months without noticeable changes in the growth rate or morphology. The morphology of our cervical keratinocytes grown in culture is shown in Figure 11. The primary keratinocytes isolated from EC tissue and grown in culture in the presence of Y-27632 are represented by only one type of small cells (Figure 11A), while keratinocytes isolated from the TZ consist of at least two types of cells that may represent keratinocytes at different differentiation states. One type appears similar to the keratinocytes isolated from the EC, while the other type is larger (Figure 11B).

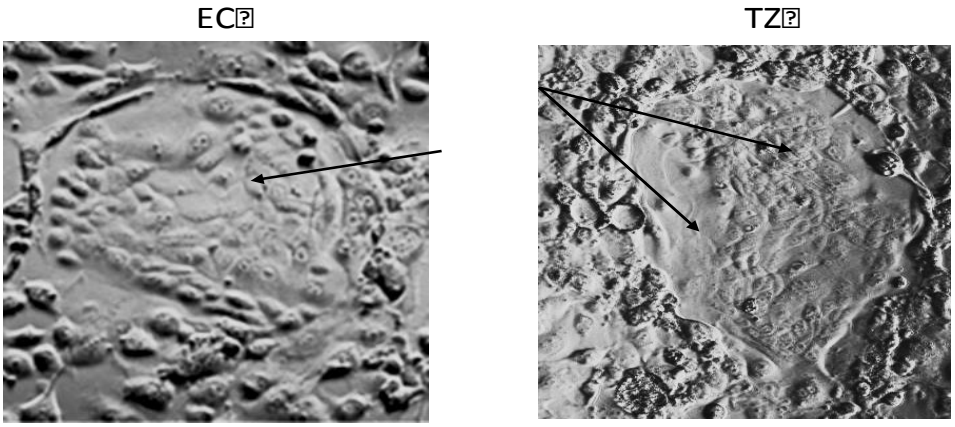

Figure 11. Morphology of cultured keratinocytes isolated from EC and TZ. Arrows indicate clusters of keratinocytes. Feeder cells can be seen around the clusters.

Variability in ROS Levels in Cultured Primary Keratinocytes Isolated from the TZ is Higher than in Keratinocytes Isolated from the EC

To assess how closely our cultured primary keratinocytes reflected the levels of ROS observed in their corresponding tissues of origin, we measured the levels of ROS in primary keratinocytes isolated from the TZ and EC regions of 5 patients. Estimation of ROS levels in cultured cells was performed following staining of cells with DCFDA (which detects H_2O_2, OH^- and ROO^-) and with DHE (which detects O_2^-) (Peshavariya et al., 2007) followed by flow cytometry. Surprisingly, the level of superoxides as detected by DHE in cultured keratinocytes was approximately the same for both EC and TZ keratinocytes, and did not vary significantly between patients (Figure 12A, grey bars). In contrast, the level of ROS as detected by DCFDA was highly variable, both between TZ and EC keratinocytes and between patients (Figure 12A, black bars). One representative flow profile of DCFDA-detected ROS from EC and TZ keratinocytes (isolated from patient #16) is presented in Figure 12B. The lowest levels of DCFDA-detected ROS were found in the EC and TZ keratinocytes isolated from patient #20, while the highest values were detected in patient #16, with corresponding differences between ROS in TZ and EC keratinocytes of approximately 1.3 and 2, respectively (Figure 12A). The difference in the levels of DCFDA-detected ROS between EC keratinocytes isolated from different women was approximately 3-fold, while the difference between TZ keratinocytes was nearly 5-fold (Figure 12A). Interestingly, the level of ROS in keratinocytes isolated from the TZ was higher than in keratinocytes isolated from the EC in all 5 specimens (Figure 12A). Because the keratinocytes were isolated, cultured and grown *in vitro* conditions, it

is likely that the observed level of ROS is largely driven by biological factors such as the cell's genetic and epigenetic background.

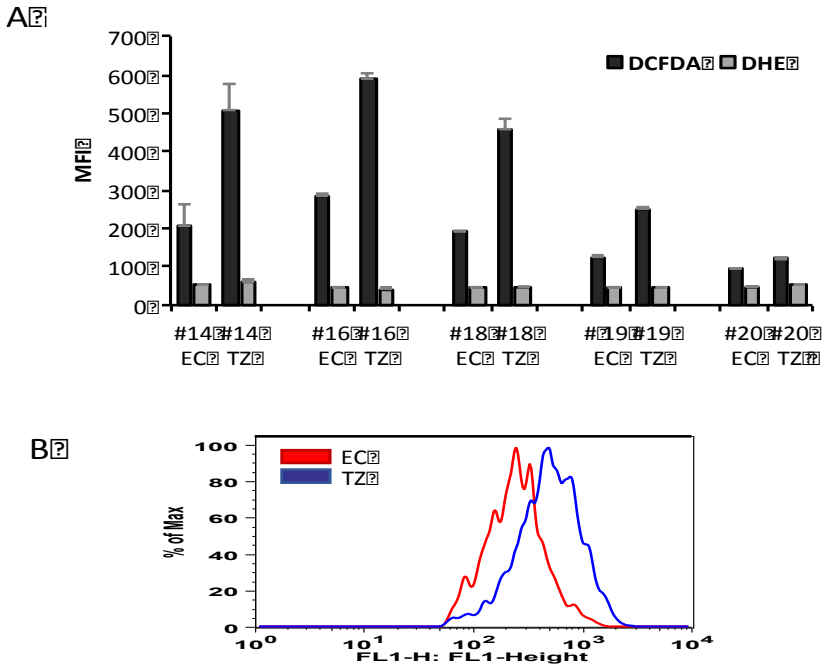

Figure 12. Cultured primary keratinocytes isolated from the TZ display higher levels of ROS than do keratinocytes isolated from the EC. **(A)** The level of ROS detected by DCFDA varies between patients, while the level of superoxides detected by DHE does not change. ROS levels were detected by flow cytometry after staining with DCFDA and DHE. Measurements were done in triplicate, and the mean fluorescent intensity (MFI) was estimated using FlowJo software. **(B)** A representative flow diagram for TZ and EC keratinocytes isolated from specimen #16.

ROS Levels in Tissue Homogenates Correspond to Those Observed in Cultured Keratinocytes

To assess how well the cultured keratinocytes reflect the primary tissue from which they were derived, we compared the levels of ROS detected in tissues with those observed in cultured keratinocytes isolated from those same tissues. The values obtained are shown in the same graph (Figure 13). These results revealed a good correlation between ROS levels in tissue and the corresponding cultured keratinocytes. To determine whether the variances in ROS levels between tissues and the corresponding cultured keratinocytes were significantly different, we employed the F-test. The F-value was equal to 1.4, a value that is lower than F-critical (3.79), thereby demonstrating that the two variables do not differ significantly and that the difference between values could be explained by random events.

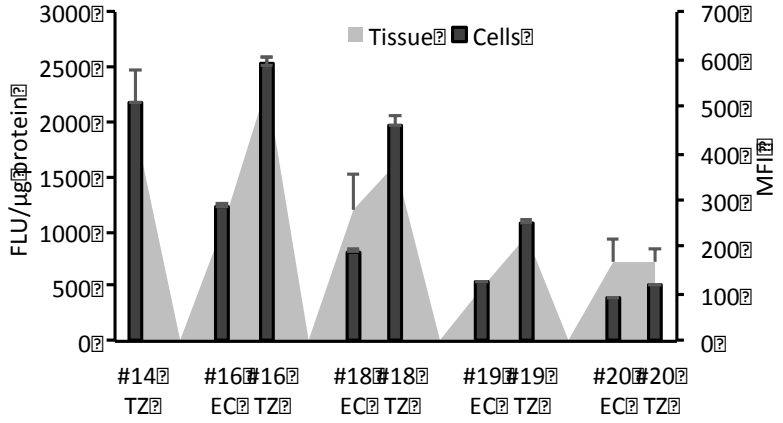

Figure 13. ROS levels in cervical tissues mirror the ROS levels observed in the corresponding cultured keratinocytes. ROS levels from TZ tissues (presented in Figure 10A) and in the corresponding cultured keratinocytes (presented in Figure 12A) are plotted on the same graph. ROS levels (in MFI) for cultured keratinocytes are shown as stacked areas, while the ROS levels in tissues (FLU/µg protein) are presented as bars. The F-test was used to determine whether the variances in ROS levels for tissues and in ROS levels for cultured keratinocytes were significantly different.

Low Levels of Gpx1/2, an Antioxidant Enzyme, Correlate with Higher Levels of ROS in TZ Keratinocytes.

ROS levels in cells reflect the functions of both pro-and anti-oxidant enzyme systems (D'Autreaux & Toledano, 2007; Ray et al., 2012). Previously, we demonstrated that the increase in ROS levels following HPV16 E6* expression is due to a reduction in the expression of SOD2 and GPX1/2 (Williams et al., 2014b). For this reason, we considered the possibility that our currently observed difference in ROS levels between TZ- and EC-derived cells could also be a result of differential expression of anti-oxidant enzymes. To test this idea, we compared the expression level of four anti-oxidant enzymes, SOD1, SOD2, Gpx ½ and Glutahione reductase (GR), in keratinocytes derived from patients #14 and #16. These two sets were chosen because they displayed significant differences in ROS levels between their TZ and EC keratinocytes (Figure 12A). Immunoblot analysis clearly demonstrated that Gpx1/2 expression was lower in TZ than in EC cells, while no significant difference in the expression of the other enzymes was observed (Figure 14). This observation is consistent with the idea that the differences in ROS observed between TZ and EC keratinocytes could be a result of differences in the expression of certain antioxidant enzymes.

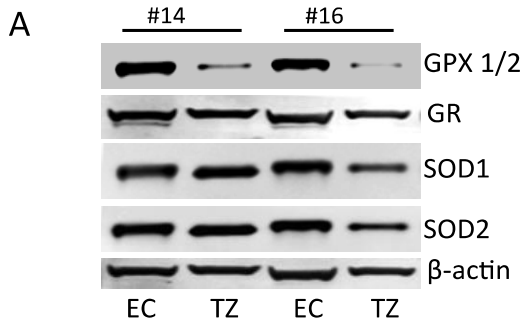

Figure 14. Decreased levels of GPX1/2 expression were observed in TZ keratinocytes as compared to EC keratinocytes. Immunoblot analysis was carried out for cultured EC and TZ cells isolated from specimens #14 and #16, and normalized to β-actin expression. GPX ½ - glutathione peroxidase, GR – glutathione reductase, SOD1 and SOD2 – superoxide dismutase 1 and 2.

High Levels of 8-Oxoguanine, a Marker of DNA Damage, Correlate with Higher Levels of ROS

One major outcome of higher levels of cellular ROS is an induction of chronic OS. OS is well known for its ability to damage multiple biomolecules, including DNA, and the oxidation-induced incorporation of 8-oxoguanine into DNA lesions can lead to mismatch mutations during DNA synthesis (Cooke et al., 2003; Olinski et al., 1992). The data shown in Figure 12 demonstrates that ROS levels in keratinocytes differ both between TZ- and EC-derived cells, and between specimens derived from different patients. The well-established linkage between OS and DNA damage therefore suggested that cells with higher levels of ROS would also display higher level of 8-oxoguanine. To test this idea, we measured the level of 8-oxoguanine in cultured keratinocytes that displayed variable levels of ROS. 8-oxoguanine levels were estimated by flow cytometry following Avidin-FITC staining, as avidin is able to bind to lesions containing 8-oxoguanine (Achanta & Huang, 2004). Figure 15 presents the measured levels of 8-oxoguanine together with DCFDA-detected levels of ROS. The highest level of ROS was detected in TZ keratinocytes isolated from specimen #16; these cells are also the ones displaying the highest level of 8-oxoguanine. We also note that cells isolated from the same patient, but derived from the EC, displayed lower levels of both ROS and 8-oxoguanine than did the corresponding TZ cells (Figure 15). The other specimens also displayed good correspondence between their levels of ROS and 8-oxoguanine (Figure 15). Furthermore, the variability observed between these two variables is not significant as estimated by the F-test. These data, therefore, are consistent with the idea that higher

levels of cellular oxidative stress, as determined by increased levels of ROS, cause a downstream increase in the level of DNA damage.

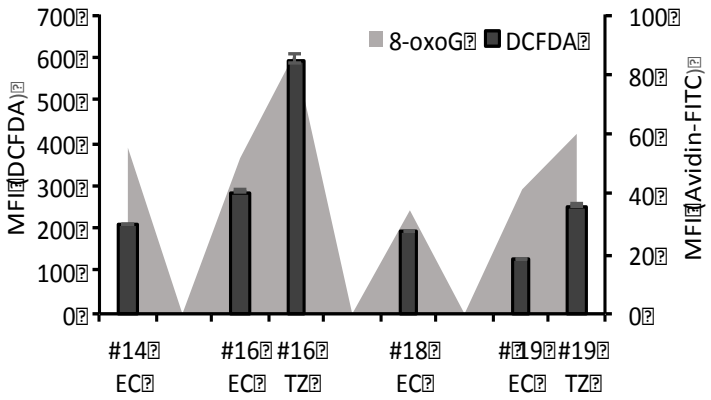

Figure 15. ROS and DNA damage levels in cervical keratinocytes closely mirror each other. DNA damage was assessed by detection of 8-oxoguanine using Avidin-FITC. Cells were fixed in 4% paraformaldehyde, washed and stained with 50 µM of Avidin-FITC for 1 h. The level of bound Avidin-FITC, presented as mean fluorescent intensity (MFI (Avidin-FITC)) was detected by flow cytometry and analyzed using FlowJo software. ROS levels were measured as described for Figure 12A. The MFI values for DNA damage (Avidin-FITC staining) and for ROS (DCFDA staining) were plotted on the same graph. To determine whether the variances in ROS levels and in 8-oxoguanine levels were significantly different, the F-test was used.

Discussion

In this report, we demonstrate for the first time a significant variability between women with regards to the background levels in ROS levels found in their cervical TZ tissue (Figure 10A). In particular, the difference between the lowest and highest levels of H_2O_2, OH^- and ROO^- species, as detected with DCFDA, was approximately 3-fold. It is generally assumed that the TZ epithelium is the site of neoplastic changes, and the most common area for the origination of cervical cancer (Herfs et al., 2012; Schiffman et al., 2007). The formation of cervical lesions may be facilitated by HPV infection of TZ cells, which can subsequently go on to form the basal layer of the stratified epithelium where HPV life cycle occurs.

The importance of our observation may lie in the possible clinical consequences of higher levels of ROS in the cervices of some woman, and in particular, how these higher levels of ROS may affect the integration of HPV. In 88% of specimens obtained from cervical cancers, HPV DNA was found integrated into the host genomes (Klaes et al., 1999). Therefore, an integration of HPV genome into that of the host is considered to function as a critical step in the development of many or most cases of cervical cancer. In many cases, the mechanism through which HPV integration leads to cancer development is thought to be a loss of functional E2 (a negative regulator of E6 and E7 expression) due to linearization, followed by increased expression of the E6 and E7 oncogenes. The resulting over-expression of E6 and E7 increases cellular proliferation at the same time that it decreases responsiveness to apoptotic signals.

Integration proceeds by way of non-homologous recombination, and therefore requires linearization of the episome and breakage of the host chromosome.

Epidemiological and molecular data both point toward OS as an important contributor to viral integration. For example, epidemiological data link conditions known to cause OS and DNA damage, such as smoking and co-infection with the STD-associated pathogens *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, with increased incidence of HPV-mediated cancers (reviewed in (Y. Chen et al., 2014; Williams et al., 2011)). Recently, we demonstrated that depletion of the antioxidant glutathione induced OS in cells and led to an increase in the frequency of HPV integration in human cervical keratinocytes that contained episomal HPV (Chen Wongworawat, Filippova, Williams, Filippov, & Duerksen-Hughes, 2016). Consistent with these proposed connections between OS, DNA damage, HPV integration and cancer, integration associated with oxidative stress has been demonstrated for another DNA virus, Hepatitis B (Cougot et al., 2005; Hu et al., 2010; Petersen et al., 1997). In the examples discussed above, the etiological factors that promote HPV-induced carcinogenesis by increasing the probability of HPV integration likely include environmental agents that can directly or indirectly increase cellular OS and the resulting DNA damage. Virus-derived factors, such as co-infection with other viruses, viral load and persistence of HPV infection can also promote HPV integration (Munoz et al., 2006). These virus-associated factors may also be connected to increased OS; recently, we demonstrated that expression of the E6 splice variant, E6*, but not the full-length version of E6, induced increases in ROS, DNA damage (Williams et al., 2014b) and the frequency of foreign DNA integration (Chen Wongworawat et al., 2016).

In addition to the exogenously-derived risk factors discussed above, Munoz *et al* (Munoz et al., 2006) have suggested that host-related factors, such as endogenous hormone levels, genetic background and factors related to the immune response, could

also contribute to cancer promotion. Logic for this suggestion comes from the fact that under normal circumstances, ROS levels are maintained in a condition of homeostasis by balanced functioning of pro- and anti-oxidant systems (D'Autreaux & Toledano, 2007; Ray et al., 2012) that are regulated by individual genetic determinants. In addition to such genetic determinants, life-style and physiological factors can also influence gene expression levels through changes to epigenetic regulation (Jaenisch & Bird, 2003). Both genetic and epigenetic factors are examples of biological factors that can contribute to the variability in levels of ROS observed in different individuals.

The evidence presented in this report indicates an important role for individual genetic and epigenetic variation in determining the overall level of cellular ROS and importantly, the resulting level of DNA damage. For the first time, we have demonstrated that individual women display significantly different levels of DNA damage in their cervical cells (Figure 15). Furthermore, the levels of ROS correlate with those of DNA damage. The higher levels of ROS seen in some women may reflect variability in mutation rates, and, if these women were to be harboring episomal HPV DNA, could result in an increased probability of integration. In addition, high levels of ROS in TZ cells infected with HPV have the potential to increase expression of the E6/E7 oncogenes (Wei et al., 2009), leading to higher level of DNA damage (Wei et al., 2014), and inhibit pro- and repress anti-tumor pathways (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006a). It is well known that OS itself is a tumor-promoting factor; in cooperation with HPV, it becomes a more potent carcinogen.

The results reported here also address the question of the relative roles played by environmental agents and other external factors *vs* internal factors such as genetic

variation and epigenetic programming. While acute OS induced by exogenously-derived factors can, in principle, be eliminated by removing the insult, the effect on cellular OS exerted by biological factors represents a more chronic and constant influence. The variability of ROS levels in cervical tissues observed in different women (Figure 10A) could, in principle, be mediated both by exogenous factors and by genetic/epigenetic factors that control the level of ROS, as the genetic and epigenetic capacity of cells to deal with oxidative stress will affect the ability of those cells to deal with both endogenous and exogenous insults. In contrast, the cultured keratinocytes from different individuals represent a model system in which variability in the influence of exogenous factors is removed because the cells are maintained in the same *in vitro* conditions for an extended period. The comparison of ROS levels between cervical tissues and cultured keratinocytes revealed a strong similarity, indicating that ROS levels in tissues are likely determined primarily by biological factors (Figures 10 and 12) since the pattern of ROS level in tissue samples was similar to the pattern seen in the corresponding primary keratinocytes (Figure 13). For example, in tissue homogenates and in cultured cells isolated from sample #16, the levels of ROS detected by two independent methods showed a similar pattern in which the level was lower in EC keratinocytes and higher in TZ keratinocytes. The slightly higher level of ROS noted in homogenates of #18 EC, #20 EC and #20 TZ as compared to the corresponding cells may be due either to the influence of exogenous factors or by random fluctuations (Figure 13). An F-test performed on the two variables favors the explanation of random fluctuations. Overall, these results suggest that the level of ROS in cervical tissues primarily determined by biological factors. This finding is important for future studies, as it provides direction toward

inquiries designed to understand why the disease is more prevalent in certain populations than in others. In addition, the close correlation between tissues and their corresponding cells indicates that further experiments focused on the role of ROS in HPV-mediated cervical carcinogenesis can be carried out using unrestricted cellular material derived from real patients.

Interestingly, a comparison of ROS levels between single- (TZ) and multilayered (EC) epithelium from cervix has not been previously reported. We found that ROS levels in TZ tissue were higher than or equal to the ROS levels found in the corresponding EC tissue (Figure 10B). A similar observation was made with the cultured keratinocytes (Figure 12B). This difference may suggest a reason that TZ tissue is the primary target for HPV infection/cervical cancer.

The difference between EC and TZ cells with regards to their ROS levels as detected by DCFDA is consistent with, and may be caused by, a differential level of expression of the antioxidant enzyme, Gpx ½ (Figure 14). While the level of superoxide-converting enzymes such as SOD1 and SOD2 (McCord & Fridovich, 1988) was unchanged, the level of Gpx ½, which converts H_2O_2 molecules to water in the presence of glutathione (McCord & Fridovich, 1988), was lower in TZ than in EC cells (Figure 14), coincident with the higher level of ROS in these cells. Interestingly, the level of superoxide radicals as detected by DHE (Figure 12A) was similar between EC and TZ cells; this is consistent with the similar levels of SOD1 and SOD2 expression observed in these cells (Figure 14). These data provide a possible mechanistic explanation of the lower level of species such as H_2O_2, OH^- and ROO^- that were observed in EC as compared to TZ cells (Figure 12A).

Overall, our findings point toward significant variability in ROS levels among women, and shed light on the potential contribution of increased levels of ROS, and the genetic/epigenetic contexts that support these increased levels, to HPV-mediated carcinogenesis. Future research will focus on exploring the possibility that high levels of ROS may predispose certain infected women – and populations - to HPV-mediated carcinogenesis. Our overall goal is to develop an understanding of the underlying mechanisms so as to develop novel and effective ways to intercept cancer development in HPV-infected individuals.

Conclusions

We characterized normal, non-cancer cervical tissue for their levels of ROS, and demonstrated a greater than three-fold variability in the levels of ROS between tissues isolated from different women. Primary keratinocytes were also isolated and cultured from the specimens, and displayed levels of ROS that were very similar to those observed in the corresponding tissue. Importantly, we demonstrated that the level of DNA damage also mirrored the level of ROS in both cultured cells. Finally, we found that differences in the level of ROS may be in part regulated by differences in the expression of antioxidant enzymes.

CHAPTER FIVE

DISCUSSION

Viruses have been demonstrated to be the causative agents of approximately 10%–15% of all cancers worldwide. When acting as carcinogenic agents, viruses utilize a variety of mechanisms to transform human cells. Their genome encodes proteins that reprogram the normal functioning of cells so as to favor virus production, and the most common outcome for this virus-induced reprogramming is the induction of genomic instability, as seen by the accumulation of point mutations, aberrations and DNA damage. However, the mechanisms causing this genomic instability differ between viruses. Genomic instability caused by viruses serves as a major step on the pathway leading to carcinogenesis and can be induced by viral infection and inflammation, by viral gene expression and by exogenously derived factors. All of these mechanisms can lead to increased oxidative stress, which is then able to damage host DNA. For some viruses, such as HBV, HPV and MCV, the pivotal step of the onset of tumorigenesis appears to be integration of the viral genome, which elevates viral oncogene expression and promotes cancer progression. Typically, cancer develops years to decades following the initial infection. This provides us with a unique opportunity for cancer interception, especially in cases where the tumorigenesis process requires virus integration.

In the case of HPV, potential cofactors that promote cervical carcinogenesis can be thought of as being classified into three groups: (1) environmental or exogenous cofactors, including hormonal contraceptives, tobacco smoking, parity, and co-infection with other sexually transmitted agents; (2) viral cofactors, such as infection by specific types, co-infection with other HPV types, HPV variants, viral load, and viral integration;

and (3) host cofactors, including endogenous hormones, genetic factors, and other factors related to the immune response (Munoz et al., 2006). Our results demonstrate that chronic OS, induced either by viral factors such as E6* or by exogenous factors, can induce DNA damage and increase the integration rate of both plasmid DNA and the HR HPV genome, and in this way contribute to cervical carcinogenesis. We also characterized normal, non-cancer cervical tissue and the corresponding primary keratinocytes for levels of ROS, and found that the values observed between primary tissue and isolated keratinocytes were very similar to each other, while displaying significant variability between patients. These findings shed light on the potential contribution of increased levels of ROS, and the genetic/epigenetic contexts that support these increased levels, to HPV-mediated carcinogenesis. In summary, we connected these three groups of potential cofactors that promote cervical carcinogenesis to a chain of events leading from the induction of OS to DNA damage and then to HPV integration, and finally, to the development of cervical cancer.

Our increasing understanding of the contributions of viral, exogenous and host cofactors to the etiology of cervical carcinogenesis, as well as of the networking of pathways involved in the transitions from OS induction, to DNA damage, to integration and finally to cervical cancer will continue to suggest prophylactic and therapeutic strategies to reduce the risk of HPV-mediated cervical cancer. However, the detailed mechanisms of OS induced DNA damage in HPV integration and carcinogenesis are still not fully understood and require further study (Figure 16).

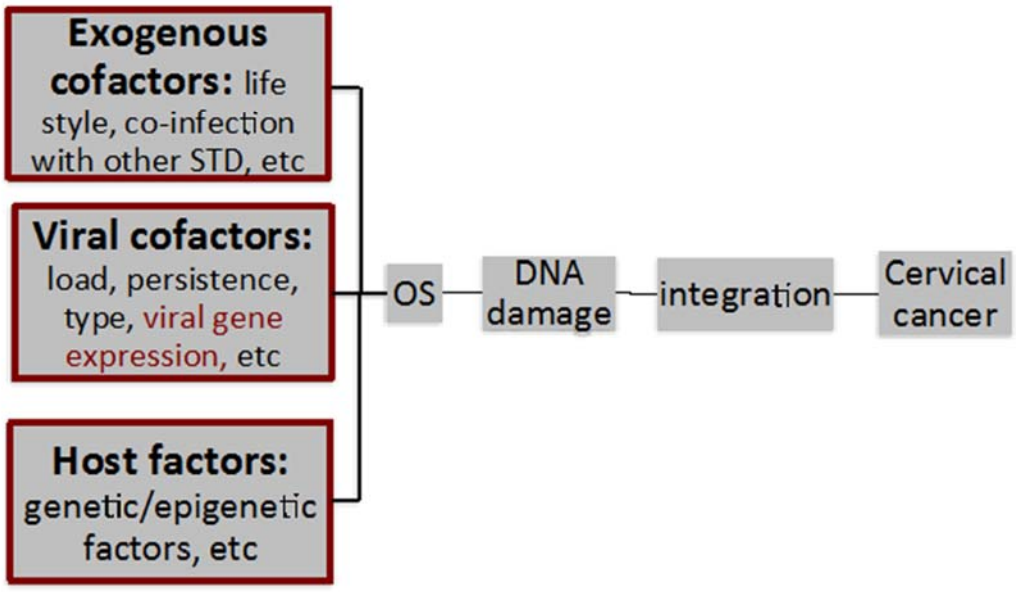

Figure 16. Potential cofactors that can promote cervical carcinogenesis. These cofactors can be classified into three groups: (1) environmental or exogenous cofactors; (2) viral cofactors; and (3) host cofactors (Munoz et al., 2006). Our results increase the understanding of the contributions of viral, exogenous and host cofactors to the etiology of cervical carcinogenesis, as well as of the networking of pathways involved in the transitions from OS induction, to DNA damage, to integration and finally to cervical cancer.

REFERENCES

Achanta, G., & Huang, P. (2004). Role of p53 in sensing oxidative DNA damage in response to reactive oxygen species-generating agents. *Cancer Res, 64*(17), 6233-6239. doi: 10.1158/0008-5472.CAN-04-0494

Adachi, M., & Ishii, H. (2002). Role of mitochondria in alcoholic liver injury. *Free Radic Biol Med, 32*(6), 487-491. doi: S0891584902007402

Agarwal, A., Saleh, R. A., & Bedaiwy, M. A. (2003). Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril, 79*(4), 829-843.

Ahn, W. S., Bae, S. M., Chung, J. E., Lee, H. K., Kim, B. K., Lee, J. M., . . . Sin, J. (2003). Evaluation of adenoassociated virus 2 and human papilloma virus 16 and 18 infection in cervical cancer biopsies. *Gynecol Oncol, 89*(1), 105-111. doi: S0090825803000088

Akagi, K., Li, J., Broutian, T. R., Padilla-Nash, H., Xiao, W., Jiang, B., . . . Gillison, M. L. (2014). Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. *Genome Res, 24*(2), 185-199. doi: 10.1101/gr.164806.113

Ali, S. H., & DeCaprio, J. A. (2001). Cellular transformation by SV40 large T antigen: interaction with host proteins. *Semin Cancer Biol, 11*(1), 15-23. doi: 10.1006/scbi.2000.0342

Altieri, F., Grillo, C., Maceroni, M., & Chichiarelli, S. (2008). DNA damage and repair: from molecular mechanisms to health implications. *Antioxid Redox Signal, 10*(5), 891-937. doi: 10.1089/ars.2007.1830

Appleby, P., Beral, V., Berrington de Gonzalez, A., Colin, D., Franceschi, S., Goodhill, A., . . . Sweetland, S. (2007). Cervical cancer and hormonal contraceptives: collaborative reanalysis of individual data for 16,573 women with cervical cancer and 35,509 women without cervical cancer from 24 epidemiological studies. *Lancet, 370*(9599), 1609-1621. doi: S0140-6736(07)61684-5

Arends, M. J., Buckley, C. H., & Wells, M. (1998). Aetiology, pathogenesis, and pathology of cervical neoplasia. *J Clin Pathol, 51*(2), 96-103.

Armstrong, J. S., Steinauer, K. K., Hornung, B., Irish, J. M., Lecane, P., Birrell, G. W., . . . Knox, S. J. (2002). Role of glutathione depletion and reactive oxygen species generation in apoptotic signaling in a human B lymphoma cell line. *Cell Death Differ, 9*(3), 252-263. doi: 10.1038/sj.cdd.4400959

Ault, K. A. (2007). Human papillomavirus vaccines and the potential for cross-protection between related HPV types. *Gynecol Oncol, 107*(2 Suppl 1), S31-33.

Autier, P., Coibion, M., Huet, F., & Grivegnee, A. R. (1996). Transformation zone location and intraepithelial neoplasia of the cervix uteri. *Br J Cancer, 74*(3), 488-490.

Bailey, S. M., & Cunningham, C. C. (2002). Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. *Free Radic Biol Med, 32*(1), 11-16. doi: S0891584901007699

Baker, C. C., Phelps, W. C., Lindgren, V., Braun, M. J., Gonda, M. A., & Howley, P. M. (1987). Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J Virol, 61*(4), 962-971.

Band, V., Zajchowski, D., Kulesa, V., & Sager, R. (1990). Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. *Proc Natl Acad Sci U S A, 87*(1), 463-467.

Banik, U., Bhattacharjee, P., Ahamad, S. U., & Rahman, Z. (2011). Pattern of epithelial cell abnormality in Pap smear: A clinicopathological and demographic correlation. *Cytojournal, 8*, 8. doi: 10.4103/1742-6413.80527

Barzon, L., Lavezzo, E., Militello, V., Toppo, S., & Palu, G. (2011). Applications of next-generation sequencing technologies to diagnostic virology. *Int J Mol Sci, 12*(11), 7861-7884. doi: 10.3390/ijms12117861

Basu, B., Yap, T. A., Molife, L. R., & de Bono, J. S. (2012). Targeting the DNA damage response in oncology: past, present and future perspectives. *Curr Opin Oncol, 24*(3), 316-324. doi: 10.1097/CCO.0b013e32835280c6

Bechtold, V., Beard, P., & Raj, K. (2003). Human papillomavirus type 16 E2 protein has no effect on transcription from episomal viral DNA. *J Virol, 77*(3), 2021-2028.

Beckmann, C. R. B., & American College of Obstetricians and Gynecologists. (2014). *Obstetrics and gynecology* (7th ed.). Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Bergsagel, D. J., Finegold, M. J., Butel, J. S., Kupsky, W. J., & Garcea, R. L. (1992). DNA sequences similar to those of simian virus 40 in ependymomas and choroid plexus tumors of childhood. *N Engl J Med, 326*(15), 988-993. doi: 10.1056/NEJM199204093261504

Bermudez-Morales, V. H., Peralta-Zaragoza, O., Guzman-Olea, E., Garcia-Carranca, A., Bahena-Roman, M., Alcocer-Gonzalez, J. M., & Madrid-Marina, V. (2009). HPV 16 E2 protein induces apoptosis in human and murine HPV 16 transformed epithelial cells and has antitumoral effects in vivo. *Tumour Biol, 30*(2), 61-72. doi: 10.1159/000214438

Bernard W. Stewart, C. P. W. (2014). Cancers of the female reproductive organs. *World Cancer Report 2014*, 465-481.

Bill, C. A., & Summers, J. (2004). Genomic DNA double-strand breaks are targets for hepadnaviral DNA integration. *Proc Natl Acad Sci U S A, 101*(30), 11135-11140. doi: 10.1073/pnas.0403925101

Bjelakovic, G., Nikolova, D., Simonetti, R. G., & Gluud, C. (2004). Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. *Lancet, 364*(9441), 1219-1228. doi: S0140673604171389

Blumberg, B. S., Alter, H. J., & Visnich, S. (1965). A "New" Antigen in Leukemia Sera. *JAMA, 191*, 541-546.

Bocchetta, M., Miele, L., Pass, H. I., & Carbone, M. (2003). Notch-1 induction, a novel activity of SV40 required for growth of SV40-transformed human mesothelial cells. *Oncogene, 22*(1), 81-89. doi: 10.1038/sj.onc.1206097

Bonilla Guerrero, R., & Roberts, L. R. (2005). The role of hepatitis B virus integrations in the pathogenesis of human hepatocellular carcinoma. *J Hepatol, 42*(5), 760-777. doi: S0168-8278(05)00091-7

Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, A., Scheurlen, W., & zur Hausen, H. (1984). A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J, 3*(5), 1151-1157.

Braun, L., Mead, J. E., Panzica, M., Mikumo, R., Bell, G. I., & Fausto, N. (1988). Transforming growth factor beta mRNA increases during liver regeneration: a possible paracrine mechanism of growth regulation. *Proc Natl Acad Sci U S A, 85*(5), 1539-1543.

Brechot, C., Kremsdorf, D., Soussan, P., Pineau, P., Dejean, A., Paterlini-Brechot, P., & Tiollais, P. (2010). Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC): molecular mechanisms and novel paradigms. *Pathol Biol (Paris), 58*(4), 278-287. doi: 10.1016/j.patbio.2010.05.001

Brechot, C., Pourcel, C., Louise, A., Rain, B., & Tiollais, P. (1980). Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. *Nature, 286*(5772), 533-535.

Bromberg, J., & Darnell, J. E., Jr. (2000). The role of STATs in transcriptional control and their impact on cellular function. *Oncogene, 19*(21), 2468-2473. doi: 10.1038/sj.onc.1203476

Buddeberg, F., Schimmer, B. B., & Spahn, D. R. (2008). Transfusion-transmissible infections and transfusion-related immunomodulation. *Best Pract Res Clin Anaesthesiol, 22*(3), 503-517.

Burchell, A. N., Winer, R. L., de Sanjose, S., & Franco, E. L. (2006). Chapter 6: Epidemiology and transmission dynamics of genital HPV infection. *Vaccine, 24 Suppl 3*, S3/52-61. doi: S0264-410X(06)00576-7

Burkle, A. (2001). Physiology and pathophysiology of poly(ADP-ribosyl)ation. *Bioessays, 23*(9), 795-806. doi: 10.1002/bies.1115

Butel, J. S., & Lednicky, J. A. (1999). Cell and molecular biology of simian virus 40: implications for human infections and disease. *J Natl Cancer Inst, 91*(2), 119-134.

Cacciotti, P., Libener, R., Betta, P., Martini, F., Porta, C., Procopio, A., . . . Gaudino, G. (2001). SV40 replication in human mesothelial cells induces HGF/Met receptor activation: a model for viral-related carcinogenesis of human malignant mesothelioma. *Proc Natl Acad Sci U S A, 98*(21), 12032-12037. doi: 10.1073/pnas.211026798

Cadet, J., Douki, T., & Ravanat, J. L. (2010). Oxidatively generated base damage to cellular DNA. *Free Radic Biol Med, 49*(1), 9-21. doi: 10.1016/j.freeradbiomed.2010.03.025

Calleja-Macias, I. E., Kalantari, M., Allan, B., Williamson, A. L., Chung, L. P., Collins, R. J., . . . Bernard, H. U. (2005). Papillomavirus subtypes are natural and old taxa: phylogeny of human papillomavirus types 44 and 55 and 68a and -b. *J Virol, 79*(10), 6565-6569. doi: 79/10/6565

Campisi, J., Kim, S. H., Lim, C. S., & Rubio, M. (2001). Cellular senescence, cancer and aging: the telomere connection. *Exp Gerontol, 36*(10), 1619-1637. doi: S0531556501001607

Carbone, M., Pass, H. I., Miele, L., & Bocchetta, M. (2003). New developments about the association of SV40 with human mesothelioma. *Oncogene, 22*(33), 5173-5180. doi: 10.1038/sj.onc.1206552

Carbone, M., Pass, H. I., Rizzo, P., Marinetti, M., Di Muzio, M., Mew, D. J., . . . Procopio, A. (1994). Simian virus 40-like DNA sequences in human pleural mesothelioma. *Oncogene, 9*(6), 1781-1790.

Carbone, M., Rizzo, P., Grimley, P. M., Procopio, A., Mew, D. J., Shridhar, V., . . . Pass, H. I. (1997). Simian virus-40 large-T antigen binds p53 in human mesotheliomas. *Nat Med, 3*(8), 908-912.

Castellsague, X., & Munoz, N. (2003). Chapter 3: Cofactors in human papillomavirus carcinogenesis--role of parity, oral contraceptives, and tobacco smoking. *J Natl Cancer Inst Monogr*(31), 20-28.

Castle, P. E., & Giuliano, A. R. (2003). Chapter 4: Genital tract infections, cervical inflammation, and antioxidant nutrients--assessing their roles as human papillomavirus cofactors. *J Natl Cancer Inst Monogr*(31), 29-34.

Castle, P. E., Hillier, S. L., Rabe, L. K., Hildesheim, A., Herrero, R., Bratti, M. C., . . . Schiffman, M. (2001). An association of cervical inflammation with high-grade

cervical neoplasia in women infected with oncogenic human papillomavirus (HPV). *Cancer Epidemiol Biomarkers Prev, 10*(10), 1021-1027.

Cerimele, F., Battle, T., Lynch, R., Frank, D. A., Murad, E., Cohen, C., . . . Arbiser, J. L. (2005). Reactive oxygen signaling and MAPK activation distinguish Epstein-Barr Virus (EBV)-positive versus EBV-negative Burkitt's lymphoma. *Proc Natl Acad Sci U S A, 102*(1), 175-179. doi: 0408381102

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., & Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science, 266*(5192), 1865-1869.

Chen, C. J., Yang, H. I., Su, J., Jen, C. L., You, S. L., Lu, S. N., . . . Iloeje, U. H. (2006). Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA, 295*(1), 65-73. doi: 295/1/65

Chen Wongworawat, Y., Filippova, M., Williams, V. M., Filippov, V., & Duerksen-Hughes, P. J. (2016). Chronic oxidative stress increases the integration frequency of foreign DNA and human papillomavirus 16 in human keratinocytes. *American J for Cancer Res, In Press*.

Chen, Y., Williams, V., Filippova, M., Filippov, V., & Duerksen-Hughes, P. (2014). Viral carcinogenesis: factors inducing DNA damage and virus integration. *Cancers (Basel), 6*(4), 2155-2186. doi: 10.3390/cancers6042155

Cheng, L., Zhang, J., Ahmad, S., Rozier, L., Yu, H., Deng, H., & Mao, Y. (2011). Aurora B regulates formin mDia3 in achieving metaphase chromosome alignment. *Dev Cell, 20*(3), 342-352. doi: 10.1016/j.devcel.2011.01.008

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science, 244*(4902), 359-362.

Chung, Y. L., Sheu, M. L., & Yen, S. H. (2003). Hepatitis C virus NS5A as a potential viral Bcl-2 homologue interacts with Bax and inhibits apoptosis in hepatocellular carcinoma. *Int J Cancer, 107*(1), 65-73. doi: 10.1002/ijc.11303

Clifford, G. M., Rana, R. K., Franceschi, S., Smith, J. S., Gough, G., & Pimenta, J. M. (2005). Human papillomavirus genotype distribution in low-grade cervical lesions: comparison by geographic region and with cervical cancer. *Cancer Epidemiol Biomarkers Prev, 14*(5), 1157-1164. doi: 14/5/1157

Collins, A. R. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol, 26*(3), 249-261. doi: MB:26:3:249

Collot-Teixeira, S., Bass, J., Denis, F., & Ranger-Rogez, S. (2004). Human tumor suppressor p53 and DNA viruses. *Rev Med Virol, 14*(5), 301-319. doi: 10.1002/rmv.431

Colotta, F., Allavena, P., Sica, A., Garlanda, C., & Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis, 30*(7), 1073-1081. doi: 10.1093/carcin/bgp127

Conway, C., Chalkley, R., High, A., Maclennan, K., Berri, S., Chengot, P., . . . Wood, H. M. (2012). Next-generation sequencing for simultaneous determination of human papillomavirus load, subtype, and associated genomic copy number changes in tumors. *J Mol Diagn, 14*(2), 104-111. doi: 10.1016/j.jmoldx.2011.10.003

Cooke, M. S., Evans, M. D., Dizdaroglu, M., & Lunec, J. (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J, 17*(10), 1195-1214. doi: 10.1096/fj.02-0752rev

Cougot, D., Neuveut, C., & Buendia, M. A. (2005). HBV induced carcinogenesis. *J Clin Virol, 34 Suppl 1*, S75-78. doi: S1386-6532(05)80014-9

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature, 420*(6917), 860-867. doi: 10.1038/nature01322

Cozad, J. (1996). Infectious mononucleosis. *Nurse Pract, 21*(3), 14-16, 23, 27-18.

Cullen, A. P., Reid, R., Campion, M., & Lorincz, A. T. (1991). Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *J Virol, 65*(2), 606-612.

Czaja, M. J., Weiner, F. R., Flanders, K. C., Giambrone, M. A., Wind, R., Biempica, L., & Zern, M. A. (1989). In vitro and in vivo association of transforming growth factor-beta 1 with hepatic fibrosis. *J Cell Biol, 108*(6), 2477-2482.

D'Autreaux, B., & Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol, 8*(10), 813-824. doi: 10.1038/nrm2256

Dalstein, V., Riethmuller, D., Pretet, J. L., Le Bail Carval, K., Sautiere, J. L., Carbillet, J. P., . . . Mougin, C. (2003). Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. *Int J Cancer, 106*(3), 396-403. doi: 10.1002/ijc.11222

Dandri, M., Burda, M. R., Burkle, A., Zuckerman, D. M., Will, H., Rogler, C. E., . . . Petersen, J. (2002). Increase in de novo HBV DNA integrations in response to oxidative DNA damage or inhibition of poly(ADP-ribosyl)ation. *Hepatology, 35*(1), 217-223. doi: S0270913902781925

Dato, S., Crocco, P., D'Aquila, P., de Rango, F., Bellizzi, D., Rose, G., & Passarino, G. (2013). Exploring the role of genetic variability and lifestyle in oxidative stress response for healthy aging and longevity. *Int J Mol Sci, 14*(8), 16443-16472. doi: 10.3390/ijms140816443

Datta, S. D., Koutsky, L. A., Ratelle, S., Unger, E. R., Shlay, J., McClain, T., . . . Weinstock, H. (2008). Human papillomavirus infection and cervical cytology in women screened for cervical cancer in the United States, 2003-2005. *Ann Intern Med, 148*(7), 493-500. doi: 148/7/493

Day, P. M., Baker, C. C., Lowy, D. R., & Schiller, J. T. (2004). Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. *Proc Natl Acad Sci U S A, 101*(39), 14252-14257. doi: 10.1073/pnas.0404229101

De Bont, R., & van Larebeke, N. (2004). Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis, 19*(3), 169-185.

De Luca, A., Baldi, A., Esposito, V., Howard, C. M., Bagella, L., Rizzo, P., . . . Giordano, A. (1997). The retinoblastoma gene family pRb/p105, p107, pRb2/p130 and simian virus-40 large T-antigen in human mesotheliomas. *Nat Med, 3*(8), 913-916.

De Marco, F. (2013). Oxidative stress and HPV carcinogenesis. *Viruses, 5*(2), 708-731. doi: 10.3390/v5020708

De Marco, F., Bucaj, E., Foppoli, C., Fiorini, A., Blarzino, C., Filipi, K., . . . Perluigi, M. (2012). Oxidative stress in HPV-driven viral carcinogenesis: redox proteomics analysis of HPV-16 dysplastic and neoplastic tissues. *PLoS One, 7*(3), e34366. doi: 10.1371/journal.pone.0034366

Deacon, J. M., Evans, C. D., Yule, R., Desai, M., Binns, W., Taylor, C., & Peto, J. (2000). Sexual behaviour and smoking as determinants of cervical HPV infection and of CIN3 among those infected: a case-control study nested within the Manchester cohort. *Br J Cancer, 83*(11), 1565-1572. doi: 10.1054/bjoc.2000.1523

Delecluse, H. J., Bartnizke, S., Hammerschmidt, W., Bullerdiek, J., & Bornkamm, G. W. (1993). Episomal and integrated copies of Epstein-Barr virus coexist in Burkitt lymphoma cell lines. *J Virol, 67*(3), 1292-1299.

Demetriou, S. K., Ona-Vu, K., Sullivan, E. M., Dong, T. K., Hsu, S. W., & Oh, D. H. (2012). Defective DNA repair and cell cycle arrest in cells expressing Merkel cell polyomavirus T antigen. *Int J Cancer, 131*(8), 1818-1827. doi: 10.1002/ijc.27440

Di Bisceglie, A. M. (1997). Hepatitis C and hepatocellular carcinoma. *Hepatology, 26*(3 Suppl 1), 34S-38S. doi: 10.1002/hep.510260706

Di Bisceglie, A. M. (2009). Hepatitis B and hepatocellular carcinoma. *Hepatology, 49*(5 Suppl), S56-60. doi: 10.1002/hep.22962

Donato, F., Boffetta, P., & Puoti, M. (1998). A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing

hepatocellular carcinoma. *Int J Cancer, 75*(3), 347-354. doi: 10.1002/(SICI)1097-0215(19980130)75:3<347::AID-IJC4>3.0.CO;2-2

Donato, F., Tagger, A., Gelatti, U., Parrinello, G., Boffetta, P., Albertini, A., . . . Nardi, G. (2002). Alcohol and hepatocellular carcinoma: the effect of lifetime intake and hepatitis virus infections in men and women. *Am J Epidemiol, 155*(4), 323-331.

Duensing, S., Lee, L. Y., Duensing, A., Basile, J., Piboonniyom, S., Gonzalez, S., . . . Munger, K. (2000). The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proc Natl Acad Sci U S A, 97*(18), 10002-10007. doi: 10.1073/pnas.170093297

Duensing, S., & Munger, K. (2002). The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. *Cancer Res, 62*(23), 7075-7082.

Durst, M., Gissmann, L., Ikenberg, H., & zur Hausen, H. (1983). A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci U S A, 80*(12), 3812-3815.

Dyson, N., Howley, P. M., Munger, K., & Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science, 243*(4893), 934-937.

El-Serag, H. B., & Rudolph, K. L. (2007). Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology, 132*(7), 2557-2576. doi: S0016-5085(07)00799-8

Epstein, M. A., Achong, B. G., & Barr, Y. M. (1964). Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. *Lancet, 1*(7335), 702-703.

Ernst, P. B., & Gold, B. D. (2000). The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu Rev Microbiol, 54*, 615-640. doi: 10.1146/annurev.micro.54.1.615

Everett, R. D. (2006). Interactions between DNA viruses, ND10 and the DNA damage response. *Cell Microbiol, 8*(3), 365-374. doi: 10.1111/j.1462-5822.2005.00677.x

Fairley, C. K., & Read, T. R. (2012). Vaccination against sexually transmitted infections. *Curr Opin Infect Dis, 25*(1), 66-72. doi: 10.1097/QCO.0b013e32834e9aeb

Fausto, N., & Mead, J. E. (1989). Regulation of liver growth: protooncogenes and transforming growth factors. *Lab Invest, 60*(1), 4-13.

Feitelson, M. A., & Lee, J. (2007). Hepatitis B virus integration, fragile sites, and hepatocarcinogenesis. *Cancer Lett, 252*(2), 157-170. doi: S0304-3835(06)00639-2

Feitelson, M. A., Reis, H. M., Tufan, N. L., Sun, B., Pan, J., & Lian, Z. (2009). Putative roles of hepatitis B x antigen in the pathogenesis of chronic liver disease. *Cancer Lett, 286*(1), 69-79. doi: 10.1016/j.canlet.2008.12.010

Feng, H., Shuda, M., Chang, Y., & Moore, P. S. (2008). Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science, 319*(5866), 1096-1100. doi: 10.1126/science.1152586

Ferber, M. J., Thorland, E. C., Brink, A. A., Rapp, A. K., Phillips, L. A., McGovern, R., . . . Smith, D. I. (2003). Preferential integration of human papillomavirus type 18 near the c-myc locus in cervical carcinoma. *Oncogene, 22*(46), 7233-7242. doi: 10.1038/sj.onc.1207006

Filippova, M., Song, H., Connolly, J. L., Dermody, T. S., & Duerksen-Hughes, P. J. (2002). The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. *J Biol Chem, 277*(24), 21730-21739. doi: 10.1074/jbc.M200113200

Finan, R. R., Musharrafieh, U., & Almawi, W. Y. (2006). Detection of Chlamydia trachomatis and herpes simplex virus type 1 or 2 in cervical samples in human papilloma virus (HPV)-positive and HPV-negative women. *Clin Microbiol Infect, 12*(9), 927-930. doi: CLM1479

Flores, E. R., & Lambert, P. F. (1997). Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. *J Virol, 71*(10), 7167-7179.

Foddis, R., De Rienzo, A., Broccoli, D., Bocchetta, M., Stekala, E., Rizzo, P., . . . Carbone, M. (2002). SV40 infection induces telomerase activity in human mesothelial cells. *Oncogene, 21*(9), 1434-1442. doi: 10.1038/sj.onc.1205203

Fradet-Turcotte, A., Bergeron-Labrecque, F., Moody, C. A., Lehoux, M., Laimins, L. A., & Archambault, J. (2011). Nuclear accumulation of the papillomavirus E1 helicase blocks S-phase progression and triggers an ATM-dependent DNA damage response. *J Virol, 85*(17), 8996-9012. doi: 10.1128/JVI.00542-11

Franco, E. L., Villa, L. L., Sobrinho, J. P., Prado, J. M., Rousseau, M. C., Desy, M., & Rohan, T. E. (1999). Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. *J Infect Dis, 180*(5), 1415-1423. doi: 10.1086/315086

Friborg, J., Jr., Kong, W., Hottiger, M. O., & Nabel, G. J. (1999). p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature, 402*(6764), 889-894. doi: 10.1038/47266

Fujimoto, A., Totoki, Y., Abe, T., Boroevich, K. A., Hosoda, F., Nguyen, H. H., . . . Nakagawa, H. (2012). Whole-genome sequencing of liver cancers identifies

etiological influences on mutation patterns and recurrent mutations in chromatin regulators. *Nat Genet, 44*(7), 760-764. doi: 10.1038/ng.2291

Gabet, A. S., Mortreux, F., Charneau, P., Riou, P., Duc-Dodon, M., Wu, Y., . . . Wattel, E. (2003). Inactivation of hTERT transcription by Tax. *Oncogene, 22*(24), 3734-3741. doi: 10.1038/sj.onc.1206468

Garcia-Closas, R., Castellsague, X., Bosch, X., & Gonzalez, C. A. (2005). The role of diet and nutrition in cervical carcinogenesis: a review of recent evidence. *Int J Cancer, 117*(4), 629-637. doi: 10.1002/ijc.21193

Georgakilas, A. G., Mosley, W. G., Georgakila, S., Ziech, D., & Panayiotidis, M. I. (2010). Viral-induced human carcinogenesis: an oxidative stress perspective. *Mol Biosyst, 6*(7), 1162-1172. doi: 10.1039/b923958h

Giglia-Mari, G., Zotter, A., & Vermeulen, W. (2011). DNA damage response. *Cold Spring Harb Perspect Biol, 3*(1), a000745. doi: 10.1101/cshperspect.a000745

Gray, H., Williams, P. L., & Bannister, L. H. (1995). *Gray's anatomy : the anatomical basis of medicine and surgery* (38th ed.). New York: Churchill Livingstone.

Gruhne, B., Sompallae, R., Marescotti, D., Kamranvar, S. A., Gastaldello, S., & Masucci, M. G. (2009). The Epstein-Barr virus nuclear antigen-1 promotes genomic instability via induction of reactive oxygen species. *Proc Natl Acad Sci U S A, 106*(7), 2313-2318. doi: 10.1073/pnas.0810619106

Gruhne, B., Sompallae, R., & Masucci, M. G. (2009). Three Epstein-Barr virus latency proteins independently promote genomic instability by inducing DNA damage, inhibiting DNA repair and inactivating cell cycle checkpoints. *Oncogene, 28*(45), 3997-4008. doi: 10.1038/onc.2009.258

Guichard, C., Amaddeo, G., Imbeaud, S., Ladeiro, Y., Pelletier, L., Maad, I. B., . . . Zucman-Rossi, J. (2012). Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat Genet, 44*(6), 694-698. doi: 10.1038/ng.2256

Guo, M., Sneige, N., Silva, E. G., Jan, Y. J., Cogdell, D. E., Lin, E., . . . Zhang, W. (2007). Distribution and viral load of eight oncogenic types of human papillomavirus (HPV) and HPV 16 integration status in cervical intraepithelial neoplasia and carcinoma. *Mod Pathol, 20*(2), 256-266. doi: 3800737

Hafner, N., Driesch, C., Gajda, M., Jansen, L., Kirchmayr, R., Runnebaum, I. B., & Durst, M. (2008). Integration of the HPV16 genome does not invariably result in high levels of viral oncogene transcripts. *Oncogene, 27*(11), 1610-1617. doi: 10.1038/sj.onc.1210791

Hagen, T. M., Huang, S., Curnutte, J., Fowler, P., Martinez, V., Wehr, C. M., . . . Chisari, F. V. (1994). Extensive oxidative DNA damage in hepatocytes of transgenic mice

with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proc Natl Acad Sci U S A, 91*(26), 12808-12812.

Halliwell, B. (2007). Oxidative stress and cancer: have we moved forward? *Biochem J, 401*(1), 1-11. doi: BJ20061131

Hamid, N. A., Brown, C., & Gaston, K. (2009). The regulation of cell proliferation by the papillomavirus early proteins. *Cell Mol Life Sci, 66*(10), 1700-1717. doi: 10.1007/s00018-009-8631-7

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70. doi: S0092-8674(00)81683-9

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013

Haverkos, H., Rohrer, M., & Pickworth, W. (2000). The cause of invasive cervical cancer could be multifactorial. *Biomed Pharmacother, 54*(1), 54-59.

Haverkos, H. W. (2005). Multifactorial etiology of cervical cancer: a hypothesis. *MedGenMed, 7*(4), 57.

Haverkos, H. W., Soon, G., Steckley, S. L., & Pickworth, W. (2003). Cigarette smoking and cervical cancer: Part I: a meta-analysis. *Biomed Pharmacother, 57*(2), 67-77.

Hawes, S. E., & Kiviat, N. B. (2002). Are genital infections and inflammation cofactors in the pathogenesis of invasive cervical cancer? *J Natl Cancer Inst, 94*(21), 1592-1593.

Hein, J., Boichuk, S., Wu, J., Cheng, Y., Freire, R., Jat, P. S., . . . Gjoerup, O. V. (2009). Simian virus 40 large T antigen disrupts genome integrity and activates a DNA damage response via Bub1 binding. *J Virol, 83*(1), 117-127. doi: 10.1128/JVI.01515-08

Herdman, M. T., Pett, M. R., Roberts, I., Alazawi, W. O., Teschendorff, A. E., Zhang, X. Y., . . . Coleman, N. (2006). Interferon-beta treatment of cervical keratinocytes naturally infected with human papillomavirus 16 episomes promotes rapid reduction in episome numbers and emergence of latent integrants. *Carcinogenesis, 27*(11), 2341-2353. doi: bgl172

Herfs, M., Yamamoto, Y., Laury, A., Wang, X., Nucci, M. R., McLaughlin-Drubin, M. E., . . . Crum, C. P. (2012). A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. *Proc Natl Acad Sci U S A, 109*(26), 10516-10521. doi: 10.1073/pnas.1202684109

Herrera, E., & Barbas, C. (2001). Vitamin E: action, metabolism and perspectives. *J Physiol Biochem, 57*(1), 43-56.

Hickman, E. S., Moroni, M. C., & Helin, K. (2002). The role of p53 and pRB in apoptosis and cancer. *Curr Opin Genet Dev, 12*(1), 60-66. doi: S0959437X01002659

Hiraku, Y., Tabata, T., Ma, N., Murata, M., Ding, X., & Kawanishi, S. (2007). Nitrative and oxidative DNA damage in cervical intraepithelial neoplasia associated with human papilloma virus infection. *Cancer Sci, 98*(7), 964-972. doi: CAS497

Hoeijmakers, J. H. (2009). DNA damage, aging, and cancer. *N Engl J Med, 361*(15), 1475-1485. doi: 10.1056/NEJMra0804615

Hopman, A. H., Smedts, F., Dignef, W., Ummelen, M., Sonke, G., Mravunac, M., . . . Ramaekers, F. C. (2004). Transition of high-grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities. *J Pathol, 202*(1), 23-33. doi: 10.1002/path.1490

Horikawa, I., & Barrett, J. C. (2001). cis-Activation of the human telomerase gene (hTERT) by the hepatitis B virus genome. *J Natl Cancer Inst, 93*(15), 1171-1173.

Hu, X., Lin, J., Xie, Q., Ren, J., Chang, Y., Wu, W., & Xia, Y. (2010). DNA double-strand breaks, potential targets for HBV integration. *J Huazhong Univ Sci Technolog Med Sci, 30*(3), 265-270. doi: 10.1007/s11596-010-0341-8

Huang, H., Reis, R., Yonekawa, Y., Lopes, J. M., Kleihues, P., & Ohgaki, H. (1999). Identification in human brain tumors of DNA sequences specific for SV40 large T antigen. *Brain Pathol, 9*(1), 33-42.

Huang, J., Deng, Q., Wang, Q., Li, K. Y., Dai, J. H., Li, N., . . . Han, Z. G. (2012). Exome sequencing of hepatitis B virus-associated hepatocellular carcinoma. *Nat Genet, 44*(10), 1117-1121. doi: 10.1038/ng.2391

Huang, L., Snyder, A. R., & Morgan, W. F. (2003). Radiation-induced genomic instability and its implications for radiation carcinogenesis. *Oncogene, 22*(37), 5848-5854. doi: 10.1038/sj.onc.1206697

Hughes, R. A. (2000). Drug injectors and the cleaning of needles and syringes. *Eur Addict Res, 6*(1), 20-30. doi: 19005

Human papillomavirus-associated cancers - United States, 2004-2008. (2012). *MMWR Morb Mortal Wkly Rep, 61*, 258-261. doi: mm6115a2

Hwang, S. G., Lee, D., Kim, J., Seo, T., & Choe, J. (2002). Human papillomavirus type 16 E7 binds to E2F1 and activates E2F1-driven transcription in a retinoblastoma protein-independent manner. *J Biol Chem, 277*(4), 2923-2930. doi: 10.1074/jbc.M109113200

Jackson, S., & Storey, A. (2000). E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage. *Oncogene, 19*(4), 592-598. doi: 10.1038/sj.onc.1203339

Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet, 33 Suppl*, 245-254. doi: 10.1038/ng1089

Jeang, K. T., Giam, C. Z., Majone, F., & Aboud, M. (2004). Life, death, and tax: role of HTLV-I oncoprotein in genetic instability and cellular transformation. *J Biol Chem, 279*(31), 31991-31994. doi: 10.1074/jbc.R400009200

Jezek, P., & Hlavata, L. (2005). Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *Int J Biochem Cell Biol, 37*(12), 2478-2503. doi: S1357-2725(05)00199-8

Jiang, Z., Jhunjhunwala, S., Liu, J., Haverty, P. M., Kennemer, M. I., Guan, Y., . . . Zhang, Z. (2012). The effects of hepatitis B virus integration into the genomes of hepatocellular carcinoma patients. *Genome Res, 22*(4), 593-601. doi: 10.1101/gr.133926.111

Jox, A., Rohen, C., Belge, G., Bartnitzke, S., Pawlita, M., Diehl, V., . . . Wolf, J. (1997). Integration of Epstein-Barr virus in Burkitt's lymphoma cells leads to a region of enhanced chromosome instability. *Ann Oncol, 8 Suppl 2*, 131-135.

Kadaja, M., Isok-Paas, H., Laos, T., Ustav, E., & Ustav, M. (2009). Mechanism of genomic instability in cells infected with the high-risk human papillomaviruses. *PLoS Pathog, 5*(4), e1000397. doi: 10.1371/journal.ppat.1000397

Kadaja, M., Sumerina, A., Verst, T., Ojarand, M., Ustav, E., & Ustav, M. (2007). Genomic instability of the host cell induced by the human papillomavirus replication machinery. *EMBO J, 26*(8), 2180-2191. doi: 10.1038/sj.emboj.7601665

Karin, M., & Greten, F. R. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol, 5*(10), 749-759. doi: nri1703

Kawanishi, S., Hiraku, Y., Pinlaor, S., & Ma, N. (2006). Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation-related carcinogenesis. *Biol Chem, 387*(4), 365-372. doi: 10.1515/BC.2006.049

Kazim, S. N., Wakil, S. M., Khan, L. A., Hasnain, S. E., & Sarin, S. K. (2002). Vertical transmission of hepatitis B virus despite maternal lamivudine therapy. *Lancet, 359*(9316), 1488-1489. doi: S0140-6736(02)08425-8

Keasler, V. V., Hodgson, A. J., Madden, C. R., & Slagle, B. L. (2007). Enhancement of hepatitis B virus replication by the regulatory X protein in vitro and in vivo. *J Virol, 81*(6), 2656-2662. doi: JVI.02020-06

Kessis, T. D., Connolly, D. C., Hedrick, L., & Cho, K. R. (1996). Expression of HPV16 E6 or E7 increases integration of foreign DNA. *Oncogene, 13*(2), 427-431.

Kieff. E, R. A. (2007). Epstein-Barr Virus and its replication. *In Fields Virology* 2603-2654.

Kim, G. E., Kim, Y. B., Cho, N. H., Chung, H. C., Pyo, H. R., Lee, J. D., . . . Suh, C. O. (2004). Synchronous coexpression of epidermal growth factor receptor and cyclooxygenase-2 in carcinomas of the uterine cervix: a potential predictor of poor survival. *Clin Cancer Res, 10*(4), 1366-1374.

Kim, Y. T., Kim, J. W., Choi, J. S., Kim, S. H., Choi, E. K., & Cho, N. H. (2004). Relation between deranged antioxidant system and cervical neoplasia. *Int J Gynecol Cancer, 14*(5), 889-895. doi: 10.1111/j.1048-891X.2004.14526.x

Klaes, R., Woerner, S. M., Ridder, R., Wentzensen, N., Duerst, M., Schneider, A., . . . von Knebel Doeberitz, M. (1999). Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. *Cancer Res, 59*(24), 6132-6136.

Klaunig, J. E., Kamendulis, L. M., & Hocevar, B. A. (2010). Oxidative stress and oxidative damage in carcinogenesis. *Toxicol Pathol, 38*(1), 96-109. doi: 0192623309356453

Klingelhutz, A. J., Foster, S. A., & McDougall, J. K. (1996). Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature, 380*(6569), 79-82. doi: 10.1038/380079a0

Kondo, T., Nonaka, H., Miyamoto, N., Yoshida, R., Matsue, Y., Ohguchi, Y., . . . Hanaoka, M. (1985). Incidence of adult T-cell leukemia-lymphoma and its familial clustering. *Int J Cancer, 35*(6), 749-751.

Kovalchuk, A. L., Qi, C. F., Torrey, T. A., Taddesse-Heath, L., Feigenbaum, L., Park, S. S., . . . Morse, H. C., 3rd. (2000). Burkitt lymphoma in the mouse. *J Exp Med, 192*(8), 1183-1190.

Kremsdorf, D., Soussan, P., Paterlini-Brechot, P., & Brechot, C. (2006). Hepatitis B virus-related hepatocellular carcinoma: paradigms for viral-related human carcinogenesis. *Oncogene, 25*(27), 3823-3833. doi: 1209559

Kripalani-Joshi, S., & Law, H. Y. (1994). Identification of integrated Epstein-Barr virus in nasopharyngeal carcinoma using pulse field gel electrophoresis. *Int J Cancer, 56*(2), 187-192.

Kryston, T. B., Georgiev, A. B., Pissis, P., & Georgakilas, A. G. (2011). Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat Res, 711*(1-2), 193-201. doi: 10.1016/j.mrfmmm.2010.12.016

Kudoh, A., Fujita, M., Zhang, L., Shirata, N., Daikoku, T., Sugaya, Y., . . . Tsurumi, T. (2005). Epstein-Barr virus lytic replication elicits ATM checkpoint signal transduction while providing an S-phase-like cellular environment. *J Biol Chem, 280*(9), 8156-8163. doi: M411405200

Kulkarni, S., Rader, J. S., Zhang, F., Liapis, H., Koki, A. T., Masferrer, J. L., . . . Dannenberg, A. J. (2001). Cyclooxygenase-2 is overexpressed in human cervical cancer. *Clin Cancer Res, 7*(2), 429-434.

Lace, M. J., Anson, J. R., Klussmann, J. P., Wang, D. H., Smith, E. M., Haugen, T. H., & Turek, L. P. (2011). Human papillomavirus type 16 (HPV-16) genomes integrated in head and neck cancers and in HPV-16-immortalized human keratinocyte clones express chimeric virus-cell mRNAs similar to those found in cervical cancers. *J Virol, 85*(4), 1645-1654. doi: 10.1128/JVI.02093-10

Lam, N. C., Gotsch, P. B., & Langan, R. C. (2010). Caring for pregnant women and newborns with hepatitis B or C. *Am Fam Physician, 82*(10), 1225-1229. doi: d8543

Lan, K. H., Sheu, M. L., Hwang, S. J., Yen, S. H., Chen, S. Y., Wu, J. C., . . . Lee, S. D. (2002). HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. *Oncogene, 21*(31), 4801-4811. doi: 10.1038/sj.onc.1205589

Lee, J., Kim, D. H., Lee, S., Yang, Q. H., Lee, D. K., Lee, S. K., . . . Lee, J. W. (2009). A tumor suppressive coactivator complex of p53 containing ASC-2 and histone H3-lysine-4 methyltransferase MLL3 or its paralogue MLL4. *Proc Natl Acad Sci U S A, 106*(21), 8513-8518. doi: 10.1073/pnas.0902873106

Lee, S. G., & Rho, H. M. (2000). Transcriptional repression of the human p53 gene by hepatitis B viral X protein. *Oncogene, 19*(3), 468-471. doi: 10.1038/sj.onc.1203312

Leonard, S. S., Xia, C., Jiang, B. H., Stinefelt, B., Klandorf, H., Harris, G. K., & Shi, X. (2003). Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. *Biochem Biophys Res Commun, 309*(4), 1017-1026.

Levine, A. J. (2009). The common mechanisms of transformation by the small DNA tumor viruses: The inactivation of tumor suppressor gene products: p53. *Virology, 384*(2), 285-293. doi: 10.1016/j.virol.2008.09.034

Levresse, V., Renier, A., Fleury-Feith, J., Levy, F., Moritz, S., Vivo, C., . . . Jaurand, M. C. (1997). Analysis of cell cycle disruptions in cultures of rat pleural mesothelial cells exposed to asbestos fibers. *Am J Respir Cell Mol Biol, 17*(6), 660-671. doi: 10.1165/ajrcmb.17.6.2854

Li, J., Wang, X., Diaz, J., Tsang, S. H., Buck, C. B., & You, J. (2013). Merkel cell polyomavirus large T antigen disrupts host genomic integrity and inhibits cellular proliferation. *J Virol, 87*(16), 9173-9188. doi: 10.1128/JVI.01216-13

Li, M., Zhao, H., Zhang, X., Wood, L. D., Anders, R. A., Choti, M. A., . . . Kinzler, K. W. (2011). Inactivating mutations of the chromatin remodeling gene ARID2 in hepatocellular carcinoma. *Nat Genet, 43*(9), 828-829. doi: 10.1038/ng.903

Li, S., & Mao, M. (2013). Next generation sequencing reveals genetic landscape of hepatocellular carcinomas. *Cancer Lett, 340*(2), 247-253. doi: 10.1016/j.canlet.2012.09.027

Li, Z., Van Calcar, S., Qu, C., Cavenee, W. K., Zhang, M. Q., & Ren, B. (2003). A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc Natl Acad Sci U S A, 100*(14), 8164-8169. doi: 10.1073/pnas.1332764100

Lilley, C. E., Schwartz, R. A., & Weitzman, M. D. (2007). Using or abusing: viruses and the cellular DNA damage response. *Trends Microbiol, 15*(3), 119-126. doi: 10.1016/j.tim.2007.01.003

Liu, Y., Tergaonkar, V., Krishna, S., & Androphy, E. J. (1999). Human papillomavirus type 16 E6-enhanced susceptibility of L929 cells to tumor necrosis factor alpha correlates with increased accumulation of reactive oxygen species. *J Biol Chem, 274*(35), 24819-24827.

Lowy, D. R., Kirnbauer, R., & Schiller, J. T. (1994). Genital human papillomavirus infection. *Proc Natl Acad Sci U S A, 91*(7), 2436-2440.

Luber, B., Arnold, N., Sturzl, M., Hohne, M., Schirmacher, P., Lauer, U., . . . Kekule, A. S. (1996). Hepatoma-derived integrated HBV DNA causes multi-stage transformation in vitro. *Oncogene, 12*(8), 1597-1608.

Lynch, H. T., Casey, M. J., Shaw, T. G., & Lynch, J. F. (1998). Hereditary Factors in Gynecologic Cancer. *Oncologist, 3*(5), 319-338.

Magnusson, P. K., & Gyllensten, U. B. (2000). Cervical cancer risk: is there a genetic component? *Mol Med Today, 6*(4), 145-148.

Majumder, M., Ghosh, A. K., Steele, R., Ray, R., & Ray, R. B. (2001). Hepatitis C virus NS5A physically associates with p53 and regulates p21/waf1 gene expression in a p53-dependent manner. *J Virol, 75*(3), 1401-1407. doi: 10.1128/JVI.75.3.1401-1407.2001

Manda, G. N., Marina T.; Neagu, Teodora-Monica. (2009). Reactive Oxygen Species, Cancer and Anti-Cancer Therapies. *current chemical biology, 3*(1), 25.

Manju, V., Kalaivani Sailaja, J., & Nalini, N. (2002). Circulating lipid peroxidation and antioxidant status in cervical cancer patients: a case-control study. *Clin Biochem, 35*(8), 621-625. doi: S0009912002003764

Marcucci, F., & Mele, A. (2011). Hepatitis viruses and non-Hodgkin lymphoma: epidemiology, mechanisms of tumorigenesis, and therapeutic opportunities. *Blood, 117*(6), 1792-1798. doi: 10.1182/blood-2010-06-275818

Marnett, L. J. (2000). Oxyradicals and DNA damage. *Carcinogenesis, 21*(3), 361-370.

Martini, F., Iaccheri, L., Lazzarin, L., Carinci, P., Corallini, A., Gerosa, M., . . . Tognon, M. (1996). SV40 early region and large T antigen in human brain tumors, peripheral blood cells, and sperm fluids from healthy individuals. *Cancer Res, 56*(20), 4820-4825.

Martini, F., Lazzarin, L., Iaccheri, L., Vignocchi, B., Finocchiaro, G., Magnani, I., . . . Tognon, M. (2002). Different simian virus 40 genomic regions and sequences homologous with SV40 large T antigen in DNA of human brain and bone tumors and of leukocytes from blood donors. *Cancer, 94*(4), 1037-1048. doi: 10.1002/cncr.10272

Mason, W. S., Liu, C., Aldrich, C. E., Litwin, S., & Yeh, M. M. (2010). Clonal expansion of normal-appearing human hepatocytes during chronic hepatitis B virus infection. *J Virol, 84*(16), 8308-8315. doi: 10.1128/JVI.00833-10

Matsuo, T., Heller, M., Petti, L., O'Shiro, E., & Kieff, E. (1984). Persistence of the entire Epstein-Barr virus genome integrated into human lymphocyte DNA. *Science, 226*(4680), 1322-1325.

Matzner, I., Savelyeva, L., & Schwab, M. (2003). Preferential integration of a transfected marker gene into spontaneously expressed fragile sites of a breast cancer cell line. *Cancer Lett, 189*(2), 207-219. doi: S0304383502005049

McCord, J. M., & Fridovich, I. (1988). Superoxide dismutase: the first twenty years (1968-1988). *Free Radic Biol Med, 5*(5-6), 363-369.

McKinney, C. C., Hussmann, K. L., & McBride, A. A. (2015). The Role of the DNA Damage Response throughout the Papillomavirus Life Cycle. *Viruses, 7*(5), 2450-2469. doi: 10.3390/v7052450

McMullan, R., Lax, S., Robertson, V. H., Radford, D. J., Broad, S., Watt, F. M., . . . Hotchin, N. A. (2003). Keratinocyte differentiation is regulated by the Rho and ROCK signaling pathway. *Curr Biol, 13*(24), 2185-2189.

Meiring, T. L., Salimo, A. T., Coetzee, B., Maree, H. J., Moodley, J., Hitzeroth, II, . . . Williamson, A. L. (2012). Next-generation sequencing of cervical DNA detects human papillomavirus types not detected by commercial kits. *Virol J, 9*, 164. doi: 10.1186/1743-422X-9-164

Meissner, J. D. (1999). Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines. *J Gen Virol, 80 (Pt 7)*, 1725-1733.

Meyer, R., Muller, M., Beneke, S., Kupper, J. H., & Burkle, A. (2000). Negative regulation of alkylation-induced sister-chromatid exchange by poly(ADP-ribose) polymerase-1 activity. *Int J Cancer, 88*(3), 351-355. doi: 10.1002/1097-0215(20001101)88:3<351::AID-IJC5>3.0.CO;2-H

Moberg, M., Gustavsson, I., Wilander, E., & Gyllensten, U. (2005). High viral loads of human papillomavirus predict risk of invasive cervical carcinoma. *Br J Cancer, 92*(5), 891-894. doi: 10.1038/sj.bjc.6602436

Molano, M., Van den Brule, A., Plummer, M., Weiderpass, E., Posso, H., Arslan, A., . . . Group, H. P. V. S. (2003). Determinants of clearance of human papillomavirus infections in Colombian women with normal cytology: a population-based, 5-year follow-up study. *Am J Epidemiol, 158*(5), 486-494.

Moody, C. A., Fradet-Turcotte, A., Archambault, J., & Laimins, L. A. (2007). Human papillomaviruses activate caspases upon epithelial differentiation to induce viral genome amplification. *Proc Natl Acad Sci U S A, 104*(49), 19541-19546. doi: 0707947104

Moody, C. A., & Laimins, L. A. (2009). Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. *PLoS Pathog, 5*(10), e1000605. doi: 10.1371/journal.ppat.1000605

Moore, P. S., & Chang, Y. (1998). Antiviral activity of tumor-suppressor pathways: clues from molecular piracy by KSHV. *Trends Genet, 14*(4), 144-150. doi: S0168-9525(98)01408-5

Moreno, V., Bosch, F. X., Munoz, N., Meijer, C. J., Shah, K. V., Walboomers, J. M., . . . Franceschi, S. (2002). Effect of oral contraceptives on risk of cervical cancer in women with human papillomavirus infection: the IARC multicentric case-control study. *Lancet, 359*(9312), 1085-1092. doi: S0140-6736(02)08150-3

Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., & Kieff, E. (1995). The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell, 80*(3), 389-399. doi: 0092-8674(95)90489-1

Munger, K., & Phelps, W. C. (1993). The human papillomavirus E7 protein as a transforming and transactivating factor. *Biochim Biophys Acta, 1155*(1), 111-123. doi: 0304-419X(93)90025-8

Munger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., & Howley, P. M. (1989). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J, 8*(13), 4099-4105.

Munoz, N., Castellsague, X., de Gonzalez, A. B., & Gissmann, L. (2006). Chapter 1: HPV in the etiology of human cancer. *Vaccine, 24 Suppl 3*, S3/1-10. doi: 10.1016/j.vaccine.2006.05.115

Munoz, N., Franceschi, S., Bosetti, C., Moreno, V., Herrero, R., Smith, J. S., . . . Bosch, F. X. (2002). Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. *Lancet, 359*(9312), 1093-1101. doi: S0140-6736(02)08151-5

10.1016/S0140-6736(02)08151-5

Murakami, Y., Saigo, K., Takashima, H., Minami, M., Okanoue, T., Brechot, C., & Paterlini-Brechot, P. (2005). Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. *Gut, 54*(8), 1162-1168. doi: 54/8/1162

Murphy, E. L., Hanchard, B., Figueroa, J. P., Gibbs, W. N., Lofters, W. S., Campbell, M., . . . Blattner, W. A. (1989). Modelling the risk of adult T-cell leukemia/lymphoma in persons infected with human T-lymphotropic virus type I. *Int J Cancer, 43*(2), 250-253.

Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K., & Ichihara, A. (1985). Inhibitory effect of transforming growth factor-beta on DNA synthesis of adult rat hepatocytes in primary culture. *Biochem Biophys Res Commun, 133*(3), 1042-1050. doi: 0006-291X(85)91241-0

Nakatsukasa, H., Nagy, P., Evarts, R. P., Hsia, C. C., Marsden, E., & Thorgeirsson, S. S. (1990). Cellular distribution of transforming growth factor-beta 1 and procollagen types I, III, and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. *J Clin Invest, 85*(6), 1833-1843. doi: 10.1172/JCI114643

Negro, F., & Alberti, A. (2011). The global health burden of hepatitis C virus infection. *Liver Int, 31 Suppl 2*, 1-3. doi: 10.1111/j.1478-3231.2011.02537.x

Nikitin, P. A., Yan, C. M., Forte, E., Bocedi, A., Tourigny, J. P., White, R. E., . . . Luftig, M. A. (2010). An ATM/Chk2-mediated DNA damage-responsive signaling pathway suppresses Epstein-Barr virus transformation of primary human B cells. *Cell Host Microbe, 8*(6), 510-522. doi: 10.1016/j.chom.2010.11.004

Oh, S. T., Kyo, S., & Laimins, L. A. (2001). Telomerase activation by human papillomavirus type 16 E6 protein: induction of human telomerase reverse transcriptase expression through Myc and GC-rich Sp1 binding sites. *J Virol, 75*(12), 5559-5566. doi: 10.1128/JVI.75.12.5559-5566.2001

Ohshima, H., Tatemichi, M., & Sawa, T. (2003). Chemical basis of inflammation-induced carcinogenesis. *Arch Biochem Biophys, 417*(1), 3-11. doi: S0003986103002832

Olinski, R., Zastawny, T., Budzbon, J., Skokowski, J., Zegarski, W., & Dizdaroglu, M. (1992). DNA base modifications in chromatin of human cancerous tissues. *FEBS Lett, 309*(2), 193-198.

Ostor, A. G. (1993). Natural history of cervical intraepithelial neoplasia: a critical review. *Int J Gynecol Pathol, 12*(2), 186-192.

Parkin, D. M., & Bray, F. (2006). Chapter 2: The burden of HPV-related cancers. *Vaccine, 24 Suppl 3*, S3/11-25.

Pass, H. I., Mew, D. J., Carbone, M., Matthews, W. A., Donington, J. S., Baserga, R., . . . Steinberg, S. M. (1996). Inhibition of hamster mesothelioma tumorigenesis by an antisense expression plasmid to the insulin-like growth factor-1 receptor. *Cancer Res, 56*(17), 4044-4048.

Pathmanathan, R., Prasad, U., Chandrika, G., Sadler, R., Flynn, K., & Raab-Traub, N. (1995). Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein-Barr virus-infected neoplasia. *Am J Pathol, 146*(6), 1355-1367.

Peebles, K. A., Lee, J. M., Mao, J. T., Hazra, S., Reckamp, K. L., Krysan, K., . . . Dubinett, S. M. (2007). Inflammation and lung carcinogenesis: applying findings in prevention and treatment. *Expert Rev Anticancer Ther, 7*(10), 1405-1421. doi: 10.1586/14737140.7.10.1405

Peitsaro, P., Hietanen, S., Johansson, B., Lakkala, T., & Syrjanen, S. (2002). Single copy heterozygote integration of HPV 33 in chromosomal band 5p14 is found in an epithelial cell clone with selective growth advantage. *Carcinogenesis, 23*(6), 1057-1064.

Peitsaro, P., Johansson, B., & Syrjanen, S. (2002). Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol, 40*(3), 886-891.

Perz, J. F., Armstrong, G. L., Farrington, L. A., Hutin, Y. J., & Bell, B. P. (2006). The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol, 45*(4), 529-538. doi: S0168-8278(06)00297-2

Peshavariya, H. M., Dusting, G. J., & Selemidis, S. (2007). Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase. *Free Radic Res, 41*(6), 699-712. doi: 778898680

Peter, M., Rosty, C., Couturier, J., Radvanyi, F., Teshima, H., & Sastre-Garau, X. (2006). MYC activation associated with the integration of HPV DNA at the MYC locus in genital tumors. *Oncogene, 25*(44), 5985-5993. doi: 1209625

Petersen, J., Dandri, M., Burkle, A., Zhang, L., & Rogler, C. E. (1997). Increase in the frequency of hepadnavirus DNA integrations by oxidative DNA damage and inhibition of DNA repair. *J Virol, 71*(7), 5455-5463.

Pett, M., & Coleman, N. (2007). Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis? *J Pathol, 212*(4), 356-367. doi: 10.1002/path.2192

Pett, M. R., Herdman, M. T., Palmer, R. D., Yeo, G. S., Shivji, M. K., Stanley, M. A., & Coleman, N. (2006). Selection of cervical keratinocytes containing integrated HPV16 associates with episome loss and an endogenous antiviral response. *Proc Natl Acad Sci U S A, 103*(10), 3822-3827. doi: 0600078103

Piboonniyom, S. O., Duensing, S., Swilling, N. W., Hasskarl, J., Hinds, P. W., & Munger, K. (2003). Abrogation of the retinoblastoma tumor suppressor checkpoint during keratinocyte immortalization is not sufficient for induction of centrosome-mediated genomic instability. *Cancer Res, 63*(2), 476-483.

Plummer, M., Herrero, R., Franceschi, S., Meijer, C. J., Snijders, P., Bosch, F. X., . . . Munoz, N. (2003). Smoking and cervical cancer: pooled analysis of the IARC multi-centric case--control study. *Cancer Causes Control, 14*(9), 805-814.

Plummer, M., Herrero, R., Franceschi, S., Meijer, C. J., Snijders, P., Bosch, F. X., . . . Group, I. M.-c. C. C. S. (2003). Smoking and cervical cancer: pooled analysis of the IARC multi-centric case--control study. *Cancer Causes Control, 14*(9), 805-814.

Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., & Gallo, R. C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A, 77*(12), 7415-7419.

Polack, A., Hortnagel, K., Pajic, A., Christoph, B., Baier, B., Falk, M., . . . Kempkes, B. (1996). c-myc activation renders proliferation of Epstein-Barr virus (EBV)-transformed cells independent of EBV nuclear antigen 2 and latent membrane protein 1. *Proc Natl Acad Sci U S A, 93*(19), 10411-10416.

Popescu, N. C., Chen, M. C., Simpson, S., Solinas, S., & DiPaolo, J. A. (1993). A Burkitt lymphoma cell line with integrated Epstein-Barr virus at a stable chromosome modification site. *Virology, 195*(1), 248-251. doi: S0042-6822(83)71367-X

Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal, 24*(5), 981-990. doi: 10.1016/j.cellsig.2012.01.008

Recommendations for counseling persons infected with human T-lymphotrophic virus, types I and II. Centers for Disease Control and Prevention and U.S. Public Health Service Working Group. (1993). *MMWR Recomm Rep, 42*(RR-9), 1-13.

Reinson, T., Toots, M., Kadaja, M., Pipitch, R., Allik, M., Ustav, E., & Ustav, M. (2013). Engagement of the ATR-dependent DNA damage response at the human papillomavirus 18 replication centers during the initial amplification. *J Virol, 87*(2), 951-964. doi: 10.1128/JVI.01943-12

Riley, P. A. (1994). Free radicals in biology: oxidative stress and the effects of ionizing radiation. *Int J Radiat Biol, 65*(1), 27-33. doi: VC5M1EPRPTCMT1UJ

Ronco, L. V., Karpova, A. Y., Vidal, M., & Howley, P. M. (1998). Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes Dev, 12*(13), 2061-2072.

Rosen, H. R. (2011). Clinical practice. Chronic hepatitis C infection. *N Engl J Med, 364*(25), 2429-2438. doi: 10.1056/NEJMcp1006613

Rosty, C., Sheffer, M., Tsafrir, D., Stransky, N., Tsafrir, I., Peter, M., . . . Sastre-Garau, X. (2005). Identification of a proliferation gene cluster associated with HPV E6/E7 expression level and viral DNA load in invasive cervical carcinoma. *Oncogene, 24*(47), 7094-7104. doi: 10.1038/sj.onc.1208854

Sakakibara, N., Mitra, R., & McBride, A. A. (2011). The papillomavirus E1 helicase activates a cellular DNA damage response in viral replication foci. *J Virol, 85*(17), 8981-8995. doi: 10.1128/JVI.00541-11

Salganik, R. I. (2001). The benefits and hazards of antioxidants: controlling apoptosis and other protective mechanisms in cancer patients and the human population. *J Am Coll Nutr, 20*(5 Suppl), 464S-472S; discussion 473S-475S.

Saslow, D., Castle, P. E., Cox, J. T., Davey, D. D., Einstein, M. H., Ferris, D. G., . . . Garcia, F. (2007). American Cancer Society Guideline for human papillomavirus (HPV) vaccine use to prevent cervical cancer and its precursors. *CA Cancer J Clin, 57*(1), 7-28. doi: 57/1/7

Sastre-Garau, X., Peter, M., Avril, M. F., Laude, H., Couturier, J., Rozenberg, F., . . . Dupin, N. (2009). Merkel cell carcinoma of the skin: pathological and molecular evidence for a causative role of MCV in oncogenesis. *J Pathol, 218*(1), 48-56. doi: 10.1002/path.2532

Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., & Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell, 63*(6), 1129-1136. doi: 0092-8674(90)90409-8

Scheurer, M. E., Tortolero-Luna, G., & Adler-Storthz, K. (2005). Human papillomavirus infection: biology, epidemiology, and prevention. *Int J Gynecol Cancer, 15*(5), 727-746. doi: IJG246

Schiffman, M., Castle, P. E., Jeronimo, J., Rodriguez, A. C., & Wacholder, S. (2007). Human papillomavirus and cervical cancer. *Lancet, 370*(9590), 890-907. doi: 10.1016/S0140-6736(07)61416-0

Schluter, V., Meyer, M., Hofschneider, P. H., Koshy, R., & Caselmann, W. H. (1994). Integrated hepatitis B virus X and 3' truncated preS/S sequences derived from human hepatomas encode functionally active transactivators. *Oncogene, 9*(11), 3335-3344.

Sedelnikova, O. A., Redon, C. E., Dickey, J. S., Nakamura, A. J., Georgakilas, A. G., & Bonner, W. M. (2010). Role of oxidatively induced DNA lesions in human pathogenesis. *Mutat Res, 704*(1-3), 152-159. doi: 10.1016/j.mrrev.2009.12.005

Seeger, C., & Mason, W. S. (2000). Hepatitis B virus biology. *Microbiol Mol Biol Rev, 64*(1), 51-68.

Seifried, L. A., Talluri, S., Cecchini, M., Julian, L. M., Mymryk, J. S., & Dick, F. A. (2008). pRB-E2F1 complexes are resistant to adenovirus E1A-mediated disruption. *J Virol, 82*(9), 4511-4520. doi: 10.1128/JVI.02713-07

Serber, Z., Lai, H. C., Yang, A., Ou, H. D., Sigal, M. S., Kelly, A. E., . . . Dotsch, V. (2002). A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. *Mol Cell Biol, 22*(24), 8601-8611.

Sethi, S., Muller, M., Schneider, A., Blettner, M., Smith, E., Turek, L., . . . Chang-Claude, J. (1998). Serologic response to the E4, E6, and E7 proteins of human papillomavirus type 16 in pregnant women. *Am J Obstet Gynecol, 178*(2), 360-364. doi: S0002937898800264

Shafritz, D. A., Shouval, D., Sherman, H. I., Hadziyannis, S. J., & Kew, M. C. (1981). Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. Studies in percutaneous liver biopsies and post-mortem tissue specimens. *N Engl J Med, 305*(18), 1067-1073. doi: 10.1056/NEJM198110293051807

Shah, K. V. (2007). SV40 and human cancer: a review of recent data. *Int J Cancer, 120*(2), 215-223. doi: 10.1002/ijc.22425

Shi, Y., Dodson, G. E., Shaikh, S., Rundell, K., & Tibbetts, R. S. (2005). Ataxia-telangiectasia-mutated (ATM) is a T-antigen kinase that controls SV40 viral replication in vivo. *J Biol Chem, 280*(48), 40195-40200. doi: C500400200

Shimoda, R., Nagashima, M., Sakamoto, M., Yamaguchi, N., Hirohashi, S., Yokota, J., & Kasai, H. (1994). Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. *Cancer Res, 54*(12), 3171-3172.

Shivapurkar, N., Harada, K., Reddy, J., Scheuermann, R. H., Xu, Y., McKenna, R. W., . . . Gazdar, A. F. (2002). Presence of simian virus 40 DNA sequences in human lymphomas. *Lancet, 359*(9309), 851-852. doi: S0140-6736(02)07921-7

Shuda, M., Feng, H., Kwun, H. J., Rosen, S. T., Gjoerup, O., Moore, P. S., & Chang, Y. (2008). T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. *Proc Natl Acad Sci U S A, 105*(42), 16272-16277. doi: 10.1073/pnas.0806526105

Shulzhenko, N., Lyng, H., Sanson, G. F., & Morgun, A. (2014). Menage a trois: an evolutionary interplay between human papillomavirus, a tumor, and a woman. *Trends Microbiol, 22*(6), 345-353. doi: 10.1016/j.tim.2014.02.009

Smith, J. S., Green, J., Berrington de Gonzalez, A., Appleby, P., Peto, J., Plummer, M., . . . Beral, V. (2003). Cervical cancer and use of hormonal contraceptives: a systematic review. *Lancet, 361*(9364), 1159-1167. doi: S0140673603129492

Smith, J. S., Lindsay, L., Hoots, B., Keys, J., Franceschi, S., Winer, R., & Clifford, G. M. (2007). Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int J Cancer, 121*(3), 621-632. doi: 10.1002/ijc.22527

Song, S. H., Lee, J. K., Oh, M. J., Hur, J. Y., Park, Y. K., & Saw, H. S. (2006). Risk factors for the progression or persistence of untreated mild dysplasia of the uterine cervix. *Int J Gynecol Cancer, 16*(4), 1608-1613. doi: IJG634

Sprague, D. L., Phillips, S. L., Mitchell, C. J., Berger, K. L., Lace, M., Turek, L. P., & Klingelhutz, A. J. (2002). Telomerase activation in cervical keratinocytes containing stably replicating human papillomavirus type 16 episomes. *Virology, 301*(2), 247-254. doi: S0042682202915424

Stanley, M. (2006). Immune responses to human papillomavirus. *Vaccine, 24 Suppl 1*, S16-22. doi: S0264-410X(05)00951-5

Sung, W. K., Zheng, H., Li, S., Chen, R., Liu, X., Li, Y., . . . Luk, J. M. (2012). Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. *Nat Genet, 44*(7), 765-769. doi: 10.1038/ng.2295

Swindle, C. S., Zou, N., Van Tine, B. A., Shaw, G. M., Engler, J. A., & Chow, L. T. (1999). Human papillomavirus DNA replication compartments in a transient DNA replication system. *J Virol, 73*(2), 1001-1009.

Szalmas, A., & Konya, J. (2009). Epigenetic alterations in cervical carcinogenesis. *Semin Cancer Biol, 19*(3), 144-152. doi: 10.1016/j.semcancer.2009.02.011

Tai, P. C., Suk, F. M., Gerlich, W. H., Neurath, A. R., & Shih, C. (2002). Hypermodification and immune escape of an internally deleted middle-envelope

(M) protein of frequent and predominant hepatitis B virus variants. *Virology, 292*(1), 44-58. doi: 10.1006/viro.2001.1239

Takakuwa, T., Luo, W. J., Ham, M. F., Sakane-Ishikawa, F., Wada, N., & Aozasa, K. (2004). Integration of Epstein-Barr virus into chromosome 6q15 of Burkitt lymphoma cell line (Raji) induces loss of BACH2 expression. *Am J Pathol, 164*(3), 967-974. doi: S0002-9440(10)63184-7

Terrin, L., Dal Col, J., Rampazzo, E., Zancai, P., Pedrotti, M., Ammirabile, G., . . . De Rossi, A. (2008). Latent membrane protein 1 of Epstein-Barr virus activates the hTERT promoter and enhances telomerase activity in B lymphocytes. *J Virol, 82*(20), 10175-10187. doi: 10.1128/JVI.00321-08

Thorland, E. C., Myers, S. L., Gostout, B. S., & Smith, D. I. (2003). Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. *Oncogene, 22*(8), 1225-1237. doi: 10.1038/sj.onc.1206170

Tohme, R. A., & Holmberg, S. D. (2010). Is sexual contact a major mode of hepatitis C virus transmission? *Hepatology, 52*(4), 1497-1505. doi: 10.1002/hep.23808

Tollefson, A. K., Oberley-Deegan, R. E., Butterfield, K. T., Nicks, M. E., Weaver, M. R., Remigio, L. K., . . . Bowler, R. P. (2010). Endogenous enzymes (NOX and ECSOD) regulate smoke-induced oxidative stress. *Free Radic Biol Med, 49*(12), 1937-1946. doi: 10.1016/j.freeradbiomed.2010.09.022

Toyooka, S., Carbone, M., Toyooka, K. O., Bocchetta, M., Shivapurkar, N., Minna, J. D., & Gazdar, A. F. (2002). Progressive aberrant methylation of the RASSF1A gene in simian virus 40 infected human mesothelial cells. *Oncogene, 21*(27), 4340-4344. doi: 10.1038/sj.onc.1205381

Toyooka, S., Pass, H. I., Shivapurkar, N., Fukuyama, Y., Maruyama, R., Toyooka, K. O., . . . Gazdar, A. F. (2001). Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma. *Cancer Res, 61*(15), 5727-5730.

Trucco, C., Oliver, F. J., de Murcia, G., & Menissier-de Murcia, J. (1998). DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines. *Nucleic Acids Res, 26*(11), 2644-2649. doi: gkb423

Tsai, W. L., & Chung, R. T. (2010). Viral hepatocarcinogenesis. *Oncogene, 29*(16), 2309-2324. doi: 10.1038/onc.2010.36

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., & Mazur, M. (2006a). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*(1), 1-40. doi: 10.1016/j.cbi.2005.12.009

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., & Mazur, M. (2006b). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact, 160*(1), 1-40. doi: S0009-2797(05)00433-3

Veldman, T., Horikawa, I., Barrett, J. C., & Schlegel, R. (2001). Transcriptional activation of the telomerase hTERT gene by human papillomavirus type 16 E6 oncoprotein. *J Virol, 75*(9), 4467-4472. doi: 10.1128/JVI.75.9.4467-4472.2001

Veldman, T., Liu, X., Yuan, H., & Schlegel, R. (2003). Human papillomavirus E6 and Myc proteins associate in vivo and bind to and cooperatively activate the telomerase reverse transcriptase promoter. *Proc Natl Acad Sci U S A, 100*(14), 8211-8216. doi: 10.1073/pnas.1435900100

Vilchez, R. A., Madden, C. R., Kozinetz, C. A., Halvorson, S. J., White, Z. S., Jorgensen, J. L., . . . Butel, J. S. (2002). Association between simian virus 40 and non-Hodgkin lymphoma. *Lancet, 359*(9309), 817-823. doi: S0140-6736(02)07950-3

Vinokurova, S., Wentzensen, N., Kraus, I., Klaes, R., Driesch, C., Melsheimer, P., . . . von Knebel Doeberitz, M. (2008). Type-dependent integration frequency of human papillomavirus genomes in cervical lesions. *Cancer Res, 68*(1), 307-313. doi: 10.1158/0008-5472.CAN-07-2754

von Knebel Doeberitz, M. (2002). New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. *Eur J Cancer, 38*(17), 2229-2242. doi: S0959804902004628

Wallace, S. S. (1998). Enzymatic processing of radiation-induced free radical damage in DNA. *Radiat Res, 150*(5 Suppl), S60-79.

Wang, H. C., Huang, W., Lai, M. D., & Su, I. J. (2006). Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis. *Cancer Sci, 97*(8), 683-688. doi: CAS235

Wang, J., Zindy, F., Chenivesse, X., Lamas, E., Henglein, B., & Brechot, C. (1992). Modification of cyclin A expression by hepatitis B virus DNA integration in a hepatocellular carcinoma. *Oncogene, 7*(8), 1653-1656.

Wang, Q., Jia, P., & Zhao, Z. (2013). VirusFinder: software for efficient and accurate detection of viruses and their integration sites in host genomes through next generation sequencing data. *PLoS One, 8*(5), e64465. doi: 10.1371/journal.pone.0064465

Waris, G., & Ahsan, H. (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog, 5*, 14. doi: 1477-3163-5-14

Waris, G., Huh, K. W., & Siddiqui, A. (2001). Mitochondrially associated hepatitis B virus X protein constitutively activates transcription factors STAT-3 and NF-kappa B via oxidative stress. *Mol Cell Biol, 21*(22), 7721-7730. doi: 10.1128/MCB.21.22.7721-7730.2001

Wei, L., Gravitt, P. E., Song, H., Maldonado, A. M., & Ozbun, M. A. (2009). Nitric oxide induces early viral transcription coincident with increased DNA damage and

mutation rates in human papillomavirus-infected cells. *Cancer Res, 69*(11), 4878-4884. doi: 10.1158/0008-5472.CAN-08-4695

Wei, L., Griego, A. M., Chu, M., & Ozbun, M. A. (2014). Tobacco exposure results in increased E6 and E7 oncogene expression, DNA damage and mutation rates in cells maintaining episomal human papillomavirus 16 genomes. *Carcinogenesis, 35*(10), 2373-2381. doi: 10.1093/carcin/bgu156

Weitzman, M. D., Lilley, C. E., & Chaurushiya, M. S. (2010). Genomes in conflict: maintaining genome integrity during virus infection. *Annu Rev Microbiol, 64*, 61-81. doi: 10.1146/annurev.micro.112408.134016

Wentzensen, N., Ridder, R., Klaes, R., Vinokurova, S., Schaefer, U., & Doeberitz, M. (2002). Characterization of viral-cellular fusion transcripts in a large series of HPV16 and 18 positive anogenital lesions. *Oncogene, 21*(3), 419-426. doi: 10.1038/sj.onc.1205104

Wentzensen, N., Vinokurova, S., & von Knebel Doeberitz, M. (2004). Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. *Cancer Res, 64*(11), 3878-3884. doi: 10.1158/0008-5472.CAN-04-0009

WHO 2014 data: Hepatitis B. Retrieved 14.07.2014, 2014, from http://www.who.int/mediacentre/factsheets/fs204/en/

WHO 2014 data: hepatitis C. Retrieved 14.07.2014, 2014, from http://www.who.int/mediacentre/factsheets/fs164/en/

Wilkins, T., Malcolm, J. K., Raina, D., & Schade, R. R. (2010). Hepatitis C: diagnosis and treatment. *Am Fam Physician, 81*(11), 1351-1357. doi: d8247

Williams, V. M., Filippova, M., Filippov, V., Payne, K. J., & Duerksen-Hughes, P. (2014a). Human Papillomavirus Type 16 E6* Induces Oxidative Stress and DNA Damage. *J Virol, 88*(12), 6751-6761. doi: JVI.03355-13

Williams, V. M., Filippova, M., Filippov, V., Payne, K. J., & Duerksen-Hughes, P. (2014b). Human papillomavirus type 16 e6* induces oxidative stress and DNA damage. *J Virol, 88*(12), 6751-6761. doi: 10.1128/JVI.03355-13

Williams, V. M., Filippova, M., Soto, U., & Duerksen-Hughes, P. J. (2011). HPV-DNA integration and carcinogenesis: putative roles for inflammation and oxidative stress. *Future Virol, 6*(1), 45-57. doi: 10.2217/fvl.10.73

Wolf, J., Pawlita, M., Jox, A., Kohls, S., Bartnitzke, S., Diehl, V., & Bullerdiek, J. (1993). Integration of Epstein Barr virus near the breakpoint of a translocation 11;19 in a Burkitt's lymphoma cell line. *Cancer Genet Cytogenet, 67*(2), 90-94. doi: 0165-4608(93)90158-I

Wu, Y. J., Parker, L. M., Binder, N. E., Beckett, M. A., Sinard, J. H., Griffiths, C. T., & Rheinwald, J. G. (1982). The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. *Cell, 31*(3 Pt 2), 693-703.

Xing, L., & Kieff, E. (2007). Epstein-Barr virus BHRF1 micro- and stable RNAs during latency III and after induction of replication. *J Virol, 81*(18), 9967-9975. doi: JVI.02244-06

Xu, B., Chotewutmontri, S., Wolf, S., Klos, U., Schmitz, M., Durst, M., & Schwarz, E. (2013). Multiplex Identification of Human Papillomavirus 16 DNA Integration Sites in Cervical Carcinomas. *PLoS One, 8*(6), e66693. doi: 10.1371/journal.pone.0066693

Yaginuma, K., Kobayashi, H., Kobayashi, M., Morishima, T., Matsuyama, K., & Koike, K. (1987). Multiple integration site of hepatitis B virus DNA in hepatocellular carcinoma and chronic active hepatitis tissues from children. *J Virol, 61*(6), 1808-1813.

Yan, Z., Cui, K., Murray, D. M., Ling, C., Xue, Y., Gerstein, A., . . . Wang, W. (2005). PBAF chromatin-remodeling complex requires a novel specificity subunit, BAF200, to regulate expression of selective interferon-responsive genes. *Genes Dev, 19*(14), 1662-1667. doi: gad.1323805

Yasunaga, J., & Matsuoka, M. (2007). Leukaemogenic mechanism of human T-cell leukaemia virus type I. *Rev Med Virol, 17*(5), 301-311. doi: 10.1002/rmv.548

Yoo, Y. D., Ueda, H., Park, K., Flanders, K. C., Lee, Y. I., Jay, G., & Kim, S. J. (1996). Regulation of transforming growth factor-beta 1 expression by the hepatitis B virus (HBV) X transactivator. Role in HBV pathogenesis. *J Clin Invest, 97*(2), 388-395. doi: 10.1172/JCI118427

Yoshida, S., & Simbulan, C. M. (1994). Interaction of poly(ADP-ribose)polymerase with DNA polymerase alpha. *Mol Cell Biochem, 138*(1-2), 39-44.

Yoshida, T., Hanada, T., Tokuhisa, T., Kosai, K., Sata, M., Kohara, M., & Yoshimura, A. (2002). Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J Exp Med, 196*(5), 641-653.

Young, L. S., & Rickinson, A. B. (2004). Epstein-Barr virus: 40 years on. *Nat Rev Cancer, 4*(10), 757-768. doi: nrc1452

Yu, M. C., & Yuan, J. M. (2002). Epidemiology of nasopharyngeal carcinoma. *Semin Cancer Biol, 12*(6), 421-429. doi: S1044579X02000858

Yu, T., Ferber, M. J., Cheung, T. H., Chung, T. K., Wong, Y. F., & Smith, D. I. (2005). The role of viral integration in the development of cervical cancer. *Cancer Genet Cytogenet, 158*(1), 27-34. doi: S0165-4608(04)00340-1

Zerfass, K., Schulze, A., Spitkovsky, D., Friedman, V., Henglein, B., & Jansen-Durr, P. (1995). Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. *J Virol, 69*(10), 6389-6399.

Zhang, X., Zhang, L., Tian, C., Yang, L., & Wang, Z. (2014). Genetic variants and risk of cervical cancer: epidemiological evidence, meta-analysis and research review. *BJOG, 121*(6), 664-674. doi: 10.1111/1471-0528.12638

Ziech, D., Franco, R., Pappa, A., & Panayiotidis, M. I. (2011). Reactive oxygen species (ROS)--induced genetic and epigenetic alterations in human carcinogenesis. *Mutat Res, 711*(1-2), 167-173. doi: 10.1016/j.mrfmmm.2011.02.015

Zienolddiny, S., Ryberg, D., & Haugen, A. (2000). Induction of microsatellite mutations by oxidative agents in human lung cancer cell lines. *Carcinogenesis, 21*(8), 1521-1526.

zur Hausen, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology, 184*(1), 9-13.

zur Hausen, H. (2000). Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst, 92*(9), 690-698.

zur Hausen, H. (2001). Oncogenic DNA viruses. *Oncogene, 20*(54), 7820-7823. doi: 10.1038/sj.onc.1204958

zur Hausen, H., Schulte-Holthausen, H., Klein, G., Henle, W., Henle, G., Clifford, P., & Santesson, L. (1970). EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. *Nature, 228*(5276), 1056-1058.